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Injectable thermosensitive alginate/β-tricalcium phosphate/aspirin hydrogels for bone augmentation

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Abstract: In this study, an injectable and thermo-sensitive alginate/β-tricalcium phosphate hydrogel (TSAH/β-TCP) was prepared for aspirin release to a bone defect. Aspirin was dissolved into a mixture of poly(N-isopropylacrylamide) (PNIPAAm), an aminated alginate-g-PNIPAAm co-polymer, and β-TCP powders. Scanning electron microscopy showed that TSAH/β-TCP had an interconnected porous microstructure with a porosity of 86.78%. The cross-linked hydrogel released approximately 40% of the aspirin in the first 3 days and then slowly released the remainder. At a low concentration (≤100 μg/mL), aspirin did not promote cell proliferation, but enhanced the alkaline phosphatase activity, and osteocalcin (OCN) and collagen I expression of human bone marrow-derived mesenchymal stem cells. The TSAH/β-TCP/aspirin hydrogel was injected into the periosteum of the rat cranial bone, and its *in vivo* bone-forming ability was evaluated at 12 weeks. A bone morphogenetic protein 2 (BMP-2)-loaded TSAH/β-TCP hydrogel was injected as a control. Micro-computed tomography showed that the percentage of mineralized tissue in the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups were similar and significantly higher than that in the TSAH/β-TCP group. Immunohistochemical staining showed considerable expression of OCN, especially in the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups. These results suggest that the injectable TSAH/β-TCP/aspirin hydrogel has great potential for bone regeneration. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 106B: 1739–1751, 2018.

Key Words: aspirin, injectable hydrogel, β-TCP, bone regeneration

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INTRODUCTION

Massive craniofacial bone loss or a defect caused by common bone trauma, congenital anomalies, oncologic resection, or pathological degenerative bone destruction is a significant health problem that severely compromises the quality of life for millions of people. The current strategies for repairing craniofacial bone defects mainly include autologous bone grafts and allografts, which are each associated with specific limitations.[1–3] Synthetic tissue-engineered bone substitutes have achieved much success as a promising alternative approach to autologous bone grafts or allografts.[4–6] In particular, injectable hydrogels show great promise for repairing craniofacial bone defects because of their fluidity, biocompatibility, minimally invasive surgery, less pain, and ability to fill irregular defects.[7–10]

Owing to its rapid cross-linking at body temperature, a thermo-sensitive hydrogel is more advantageous than ultraviolet light-crosslinking or chemical-cross-linking hydrogels. However, the insufficient bone-forming ability of thermo-sensitive hydrogels developed thus far has limited their wider application to craniofacial bone regeneration. Therefore, enhancing the bone-forming ability of an injectable thermo-sensitive hydrogel is critical for the improvement of bone regeneration. Hydrogels with robust osteogenic ability have not yet been successfully developed. Thus, development of a rapidly cross-linkable hydrogel with osteogenic ability holds great promise for treating craniofacial bone defects or for mandibular bone augmentation in a convenient and rapid manner.

In our previous study, we found that a thermo-sensitive alginate hydrogel (TSAH) composed of 8.0 wt % poly(N-

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isopropylacrylamide) (PNIPAAm) and 8.0 wt % aminated alginate-g-PNIPAAm (AAlg-g-PNIPAAm) co-polymer had promising potential to promote bone formation.[3,11] The TSAH was injected through a mucosa tunnel into the subperiosteum of the rabbit upper alveolar bone to form apophysis and ultimately new bone tissue. Despite the potential of this hydrogel for reconstructing bone tissue, the newly formed bone-like tissue was not fully mineralized. The immature bone-like tissue at the early stage of mineralization suggested that the ability of the hydrogel to induce osteogenesis needs further improvement.

Growth factors are bioactive molecules that play a key role in bone tissue regeneration, especially the widely used bone morphogenetic proteins (BMPs).[12–14] Because of the great osteoinduction ability of BMP-2, it was approved by the US Food and Drug Administration for use in anterior lumbar spine fusion procedures, tibial non-unions, and oral maxillofacial reconstructions.[15] However, the rapid increase of BMP-2 use has been accompanied by reports regarding a series of possible side effects and complications, including induction of inflammation, ectopic bone formation, hematomas, dysphagia in cervical spinal fusions, and postoperative radiculitis, among others.[16,17] An extensive review[18] concluded that the true risk of BMP-2 treatments to patients is 10–50 times higher than that originally reported. Furthermore, BMPs are easily denatured during the multiple processes of loading on biomaterials. Thus, effort is required to develop a better delivery system for BMPs for an improved long-term effect, or to find alternative strategies.

Aspirin, as a widely used and inexpensive non-steroid anti-inflammatory drug (NSAID), may have both anti-inflammatory and osteogenesis effects. According to epidemiological studies, the regular use of aspirin may have a moderate beneficial effect on bone mineral density (BMD) in postmenopausal women by regulating bone metabolism.[19,20] Yamaza[21] showed that aspirin is capable of inhibiting the Fas antibody-induced apoptosis of mesenchymal stem cells (MSCs). In animal experiments, the same group found that treatment of aspirin significantly improved the trabecular and cortical bone density in ovariectomized mice. Other studies further showed that aspirin could regulate bone metabolism.[22–24]

Based on this background, we developed a new TSAH that contains the osteoconductive powder β-tricalcium phosphate (β-TCP) and the NSAID aspirin. We investigated the osteogenesis effect of aspirin in the TSAH/β-TCP *in vitro* using human bone marrow-derived mesenchymal stem cells (hMSCs). To test the hypothesis that the aspirin delivered by TSAH/β-TCP can improve the quality and quantity of bone formation, we compared the effects of injection of aspirin-loaded TSAH/β-TCP with those of BMP-2-loaded TSAH/β-TCP as a control through *in vivo* experiments in a rat model.

MATERIALS AND METHODS

Preparation of TSAH/β-TCP or TSAH/β-TCP/aspirin composites

The TSAH was prepared by dissolving lyophilized AAlg-g-PNIPAAm (8 wt %) and PNIPAAm (8 wt %) in phosphate-buffered saline (PBS, pH 7.4) as described in our previous studies.[3,11] The β-TCP granules (50–335 μm) sterilized with Co^{60} radiation at 25 kGy were mixed into the TSAH and stirred for another 2 h [Fig. 1(A)]. Different amounts of β-TCP (0.1 g, 0.2 g, 0.4 g) were added to 1 mL of TSAH to obtain different combinations. To prepare the TSAH/β-TCP/aspirin composite, aspirin (Sigma, St Louis, MO) was dissolved in PBS at 1 mg/mL, and then lyophilized PNIPAAm, AAlg-g-PNIPAAm, and β-TCP granules were mixed into the solution. All preparations were carried out under aseptic conditions.

Morphological observations

The TSAH/β-TCP and TSAH/β-TCP/aspirin composites were first incubated at 37°C for gelation, lyophilized, and then sectioned. The cross-section was gold-coated and observed under scanning electron microscopy (SEM; Hitachi S-4800, Japan) at 15 kV. The inner morphology of the material was observed and the injectable property was tested. According to the SEM observations and injectability, a hydrogel formulation containing 1 mL of the TSAH and 0.2 g of β-TCP was chosen for the following experiments.

The porosity, pore size distribution, and specific surface area of lyophilized TSAH/β-TCP/aspirin were tested with the mercury intrusion method using PoreMasterGT 60 (Quantachrome Instruments, Boynton Beach, FL).

Rheological analysis

Rheological measurement of TSAH/β-TCP/aspirin was performed on the Physica MCR300 modular compact rheometer (AntonPaar, Germany). The heating rate was set to 3°C/min in the range of 20–45°C. The shear rate was 6.283 rad/s. The storage modulus (G′) and loss modulus (G″) were measured as functions of temperature.

In vitro release of aspirin from TSAH/β-TCP

To study the *in vitro* release of aspirin from the hydrogel, 100 μL of TSAH/β-TCP/aspirin was injected into 1.5-mL capped centrifuge tubes and gelled at 37°C. Then, 100 μL of PBS was added to each tube and incubated at 37°C for 7 days with gentle shaking. At scheduled intervals (1, 3, 5, and 7 days), the supernatant (100 μL) was collected and replaced with the same amount of fresh PBS. Using a Nanodrop 8000 ultraviolet-visible spectrophotometer (Thermo, USA), an absorption peak at 270 nm was found corresponding to aspirin. We then measured the absorbance of standard concentrations of aspirin at 270 nm respectively and performed regression analysis to make a standard curve. The amount of aspirin in the collected supernatant was determined by a spectrometric method according to the standard curve.

Cell proliferation *in vitro*

hMSCs from the redundant cancellous bone amputated during an orthognathic surgery were seeded into 96-well plates (5×10^3 cells/well) and incubated in cell culture medium [alpha-minimal essential medium (α-MEM) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL

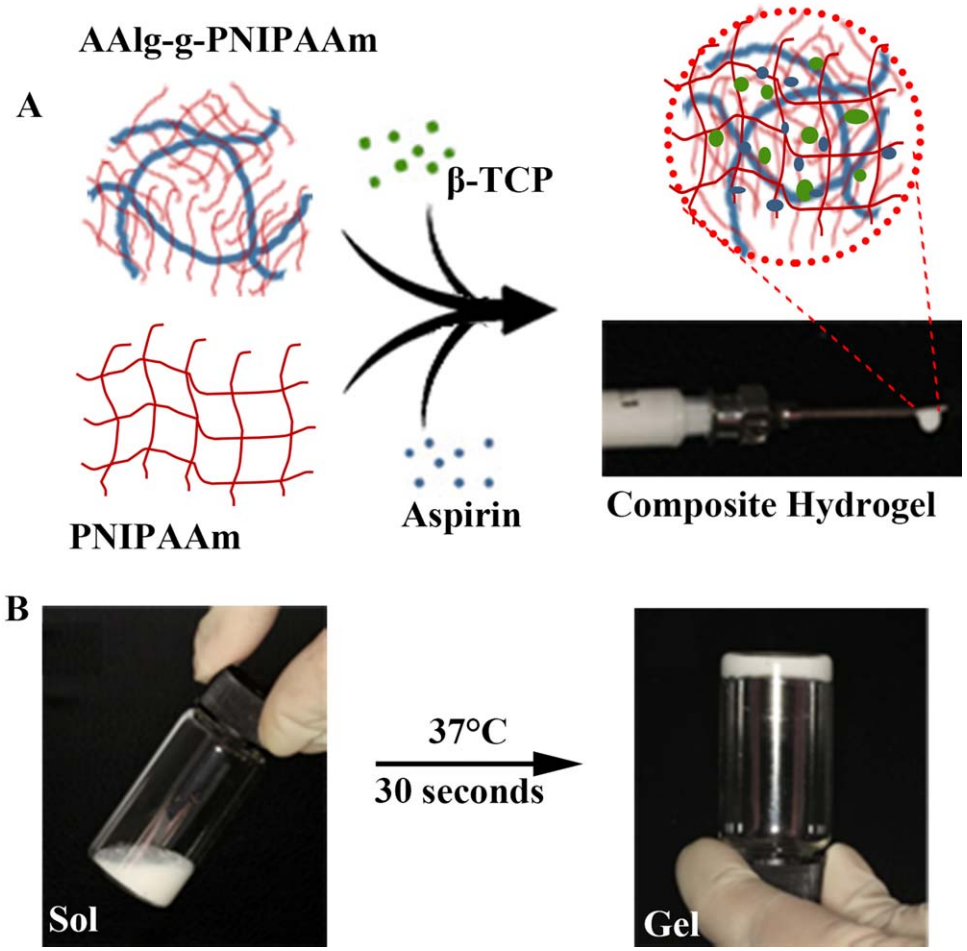

FIGURE 1. The schematic shows the preparation of the composite hydrogel TSAH/β-TCP (A). The TSAH/β-TCP is injectable at room temperature, while it becomes crosslinked hydrogel above 37°C (B).

streptomycin] containing different concentrations of aspirin (0, 50, 100, 200, 400 μg/mL) in a humidified atmosphere with 5% CO_2 at 37°C. After culture for 24, 48, and 72 h, the proliferation of hMSCs was assessed by a CCK-8 assay (Dojindo, Kumamoto-ken, Japan). The optical densities (ODs) were determined photometrically with a spectrometer at 450 nm[25].

Osteogenic differentiation and osteogenic marker expression

Alkaline phosphatase (ALP) is assumed to reflect the degree of the early osteogenic differentiation of hMSCs. To measure ALP activity of the hMSCs, the cells were plated in 48-well plates (2×10^4 cells/well) and treated with osteogenic medium (100 n*M* dexamethasone [Dex], 10 m*M* sodium β-glycerophosphate, and 10 n*M* L-ascorbic acid in growth medium) containing different concentrations of aspirin (0, 50, and 100 μg/mL). The culture medium was changed after 24 h and then every 3 days. After culture for 7 days, the cells were lysed with 0.5 mL of 1% Triton X-100 combined with repeated unfreezing. The cell lysates were then centrifuged and the supernatant was collected for ALP analysis with an ALP activity kit (Jiancheng, Nanjing, China) according to the manufacturer's protocol.

To investigate the effect of released aspirin on cell differentiation, 100 μL of TSAH/β-TCP/aspirin with 1 mg/mL loaded aspirin was injected into 1.5-mL capped centrifuge tubes and gelled at 37°C. Then, 100 μL of PBS was added to each tube and incubated on a rotary shaker (40 rpm) at 37°C for 7 days. At scheduled intervals (3 and 7 days), 100 μL of the supernatant was collected and replaced with an equal amount of fresh PBS. The supernatant mixed with α-MEM at a ratio of 1:9 (v/v) was added to hMSCs cells plated in 48-well plates (2×10^4 cells/well). As a control group, α-MEM containing 10% of the days 3 and 7 supernatants from the TSAH/β-TCP hydrogel without aspirin was used. The culture medium was changed after 24 h and then every 3 days. After 7 days, the ALP activity was detected as described above.

To further determine the osteogenic potential of the hydrogel, the expression of the osteogenesis-related markers collagen I (Col I) and osteocalcin (OCN) was detected in hMSCs. The hMSCs were seeded in 6-well plates (2×10^5 cells/well), and then treated with osteogenic media containing different concentrations of aspirin (0, 50, 100, 200 μg/

TABLE I. Quantitative PCR primers

Gene	Forward primers	Reverse primers
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA
OCN	CCTGAAAGCCGATGTGGT	AGGGCAGCGAGGTAGTGA
Col I	CCCCTGGAAAGAATGGAGAT	AATCCTCGAGCACCCTGAG

mL) the following day and continued to culture for 21 days. The culture medium was changed every 3 days. Total RNA was extracted using TRIzol (Invitrogen, USA). Complementary DNA was reverse-transcribed using 2 μg of RNA as a template and 20 μL Moloney murine leukemia virus reverse transcriptase (Promega, USA). Real-time polymerase chain reaction (PCR) was performed in duplicate using 0.5 μL complementary DNA as the template in a 20-μL reaction volume for amplification with 200 nmol/L of each primer (Table I). The thermal cycling conditions were as follows: 50°C for 2 min—95°C for 10 min—40 cycles of 94°C for 15 s—60°C for 1 min. Reactions were performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) and FastStart Universal SYBR Green PCR master mix (Roche Applied Science, USA). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

The ability of aspirin in inducing osteogenic differentiation of hMSCs *in vitro* in the absence of Dex was further detected. The hMSCs were plated in 48-well plates (2×10^4 cells/well) and treated with osteogenic medium (100 n*M* Dex, 10 m*M* sodium β-glycerophosphate, and 10 n*M* L-ascorbic acid in growth medium) containing different concentrations of aspirin (0, 100 μg/mL). In the control group, the hMSCs were treated with aspirin of 100 μg/mL without Dex. After 7 days, the ALP activity was detected as described earlier.

In vivo bone augmentation

Twenty male Sprague-Dawley rats, weighing 160–180 g, were allowed to acclimatize for 1 week in the laboratory animal housing conditions prior to the operation. All experiment protocols were approved by the Biomedical Ethical Committees of Peking University (LA2011-029). The rats were divided into the following groups according to the materials injected: (1) TSAH/β-TCP ($n = 6$), (2) TSAH/β-TCP/BMP-2 ($n = 6$; with 50 μg/mL BMP-2), (3) TSAH/β-TCP/aspirin ($n = 6$; with 1 mg/mL aspirin), (4) PBS as a blank control ($n = 2$). The rats were anesthetized by intraperitoneal injection with 1% pentobarbital sodium (40 mg/kg) and placed in prone position. The rats were then shaved and the cranial surface was disinfected. An incision of 4–5 mm was made into the skin along the midline of the cranial bone and the periosteum was exposed. A needle of 1.2 mm in diameter attached to a 1-mL syringe was inserted under the bone periosteum and shifted for 10 mm toward the face. The periosteum was carefully elevated by the tip of the needle in both the left and right direction to create a subperiosteal space with dimension of $10 \times 6 \times 3$ mm, and then 100 μL of the material was injected into the created space. After 1 min for gelation, the needle was extracted and the mucosa incision was closed with a suture. There was no need to suture the periosteum.[26] All rats were monitored daily and euthanized at 12 weeks after the surgery. Cranial bone samples were harvested and fixed in 10% neutral buffered formalin for 3 days. All specimens were analyzed by micro-computed tomography (micro-CT), histological staining, and immunohistochemistry.

Micro-CT analysis

The gross samples were scanned by micro-CT (Siemens Inveon, Germany) to determine the bone volume (X-ray source 80 kV/500 μA; exposure time 500 ms). Three-dimensional reconstruction was performed using Inveon Research Workplace (Siemens Inveon, Germany). The BMD and bone volume fraction (bone volume/total volume, BV/TV) were also determined.

Histological and immunohistochemical staining

The samples were decalcified completely in 20% ethylenediaminetetraacetic acid (pH = 7.2), embedded in paraffin wax, and cut into 4-μm-thick sections with a microtome (Leika, Germany). Conventional hematoxylin-eosin (HE) and Masson staining were then performed. The expression of the osteoblast differentiation marker OCN was examined by immunohistochemical staining (ab13420, Abcam, UK). The expression of the vascular endothelium cell marker CD34 was also examined by immunohistochemical staining (ab81289, Abcam). Three slides for each sample were chosen, and random views were selected to conduct semi-quantitative analysis of the positive staining areas by calculating the average OD (integrated OD/tissue area) using Image Pro Plus.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Among-group differences were compared using one-way analysis of variance and Turkey's multiple comparison test. The differences between groups and time points were considered statistically significant at $p < 0.05$.

RESULTS

Characterization of the TSAH/β-TCP composite scaffold

Figure 1(A) shows an overview of the preparation procedure for the TSAH/β-TCP/aspirin composite. β-TCP and aspirin were mixed into the thermo-sensitive mixture solution of PNIPAAm and AAlg-g-PNIPAAm (a comb-like thermosensitive copolymer[3]). The TSAH/β-TCP system could be injected through a needle of 1.2 mm in diameter at room temperature [Fig. 1(A)]. When the temperature increased to

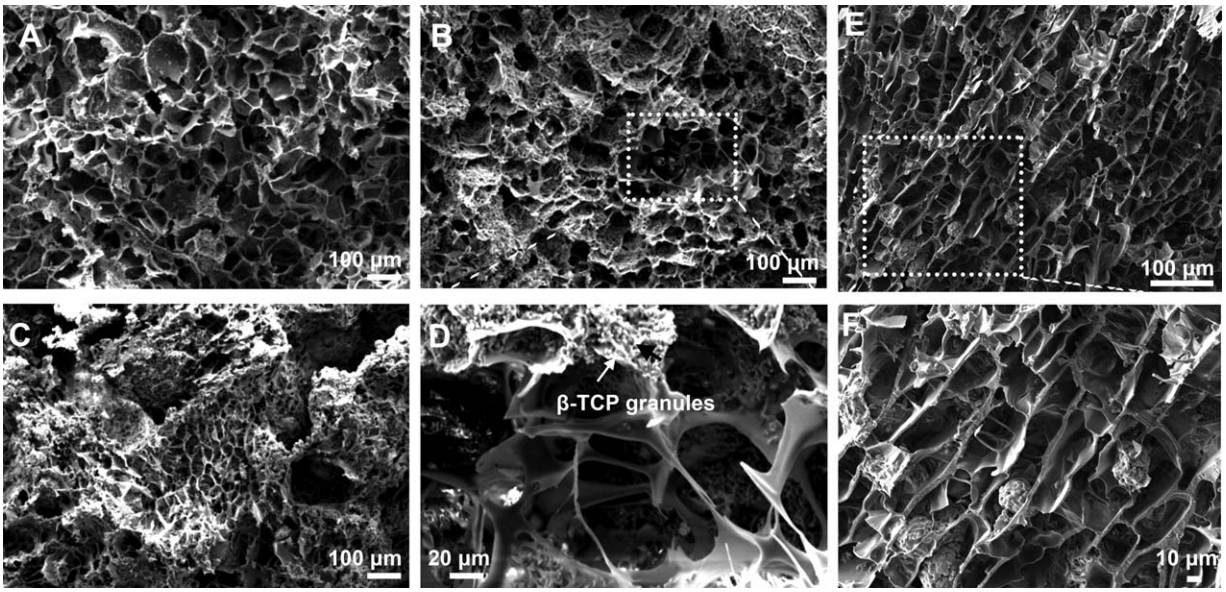

FIGURE 2. SEM images showed an interconnected porous microstructure of TSAH/β-TCP, like a honeycomb, with different contents of β-TCP granules distributed: (A) 0.1 g, (B) 0.2 g, and (C) 0.4 g. The pore size became smaller with more β-TCP granules in the hydrogel. β-TCP granules distributed in the struts of the interconnected pores (D). The addition of aspirin did not change microstructure of the system (E-F).

37°C, it gelled and gained sufficient strength (Fig. 1B). The gelation time was very rapid, at around 30 s. With 0.2 g β-TCP, the system still demonstrated great fluidity, but the injectability became worse with increasing amounts of β-TCP added. SEM observations showed that the TSAH/β-TCP hydrogel with 0.2 g β-TCP was porous [Fig. 2(B)], and the pore size decreased when the β-TCP content was increased to 0.4 g [Fig. 2(C)]. With 0.2 g of β-TCP, the pore sizes were homogenous and the pores were well interconnected [Fig. 2(D)]. The addition of aspirin did not appear to change the injectability or microstructure of the hydrogel [Fig. 2(E,F)].

The mercury porosimetry test showed that the total porosity of TSAH/β-TCP/aspirin was 86.78%. The percentage of micropores (<10 μm) was 14.5% and that of macropores (>100 μm) was 12.1%. The remaining moderately sized pores (10–100 μm) accounted for 73.4% of all pores. The specific surface area was 7.21 m^2/g.

The thermo-sensitive property of TSAH/β-TCP/aspirin was further confirmed by rheological analysis. As shown in Figure 3(A), the hydrogel had a low elastic modulus (G′) and viscous modulus (G″) below 32°C, indicating that the system was flowable. As the temperature increased, the G′ and G″ values increased markedly and reached the maximum at 37°C, indicating that the system became gelled.

Release profile of aspirin from the TSAH/β-TCP *in vitro*

The cumulative released percentage of aspirin from the supernatant is shown in Figure 3(B). The release of aspirin from TSAH/β-TCP before the first 3 days showed a first-order-like release profile. Approximately 20% of the aspirin was released from TSAH/β-TCP the first day and then 40% was released over the following 3 days. The release gradually slowed down at 5 days to the end of the experiment at day 7.

Low aspirin concentration enhanced osteogenic markers of hMSCs *in vitro*

The results of the CCK-8 assay showed that aspirin at a low concentration (<200 μg/mL) had no significant effect on the proliferation of hMSCs during the experimental period

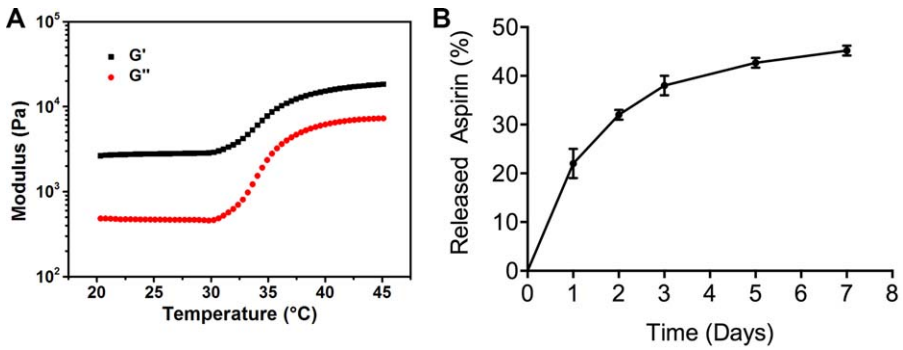

FIGURE 3. Changes of storage modulus (G′), loss modulus (G″) of the TSAH/β-TCP/aspirin upon heating process in the rheological analysis (A). *In vitro* release measurement of aspirin showed that the release of aspirin from the hydrogel into the supernatant decreased over time (B).

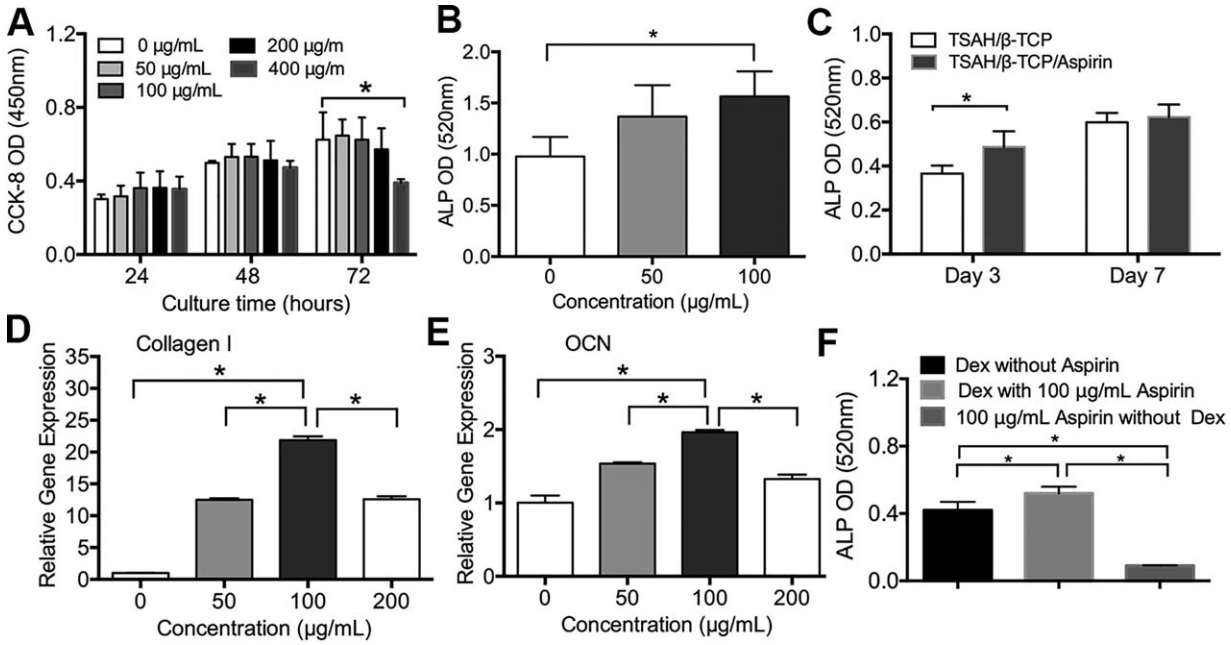

FIGURE 4. Cell proliferation assay showed that aspirin at low concentration (<200 μg/mL) had no effect on the cell proliferation or viability of hMSCs (A), but aspirin at 100 μg/mL promoted the ALP activity of hMSCs after 7 days (B). hMSCS cultured with the 3rd day's supernatant of TSAH/β-TCP/aspirin hydrogel significantly promoted the ALP activity (C). Aspirin at 100 μg/mL promoted the expression of Col I (D) and OCN (E). The ALP activity of hMSCs cultured without Dex was significantly lower than that with Dex, with or without aspirin (F).

(72 h), which showed a tendency to decline. However, the cell viability of hMSCs was significantly lower under a high aspirin concentration (400 μg/mL) than that of other groups on the third day [$p < 0.05$, Figure 4(A)].

The ALP activity of hMSCs cultured with 100 μg/mL aspirin was significantly higher than that detected in the negative control group ($p < 0.05$), although there was no effect of 50 μg/mL aspirin on the ALP activity of hMSCs [Fig. 4(B)]. When the hMSCs were cultured with the 3rd day's supernatant of TSAH/β-TCP/aspirin, ALP production in the released aspirin supernatant was significantly higher than that in the TSAH/β-TCP supernatant [$p < 0.05$, Fig. 4(C)]. However, there was no significant difference in ALP activity between the supernatants of the two groups over time.

The quantitative PCR results showed that the hMSCs cultured with 100 μg/mL aspirin expressed significantly more Col I and OCN compared with the other groups [$p < 0.05$; Fig. 4(D,E)].

The ALP activity of hMSCs cultured in the medium without Dex was significantly lower than any group with Dex (with or without aspirin). Consistent with the former result, in the presence of Dex, the ALP activity of hMSCs cultured with 100 μg/mL aspirin was significantly higher than that without aspirin [$p < 0.05$, Fig. 4(F)].

Aspirin and BMP-2 enhanced bone regeneration *in vivo*

The TSAH/β-TCP solution formed a gel at ~1 min after injection under the bone periosteum [Fig. 5A,B)]. No rat presented any sign of pain, distress, or infection.

Micro-CT analysis showed that the bone height was markedly augmented in all three hydrogel groups compared to that of the PBS control group [Fig. 5(C)]. The cross-sectioned and three-dimensional reconstructed images demonstrated that regenerated bone with a higher density mainly formed along the edge of the protuberant area and at the periphery of the β-TCP granules. In the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups, denser mineralized tissue was found along the original bone surface and β-TCP granules than that detected in the TSAH/β-TCP group, with no difference between the BMP-2- and aspirin-loaded TSAH/β-TCP groups. Moreover, all hydrogel groups showed augmented cranial bones. In particular, the percentages of mineralized tissue (BV/TV) in the TSAH/β-TCP/BMP-2 ($46.68 \pm 2.70\%$) and TSAH/β-TCP/aspirin ($51.42 \pm 6.26\%$) groups were similar, but were significantly higher than that in the TSAH/β-TCP group [$38.06 \pm 4.20\%$, $p < 0.05$; Fig. 6(A)]. Similarly, the BMD values of the regenerated tissue did not differ between the TSAH/β-TCP/BMP-2 (1182.36 ± 6.52 mg/cc) and TSAH/β-TCP/aspirin (1186.95 ± 15.73 mg/cc) groups, but were significantly higher than that in the TSAH/β-TCP group [1162.54 ± 5.80 mg/cc, $p < 0.05$, Fig. 6(B)].

Aspirin- and BMP-2-loaded hydrogels enhanced collagen deposition and osteogenesis

HE staining showed that the majority of the material was degraded and new tissues grew into the transplant areas at 12 weeks in all groups (Fig. 7). Multinucleated giant cells were found adjacent to the remnant TSAH, which may participate in the degradation of the material. A large amount of collagen fibers stained pink grew into the implantation

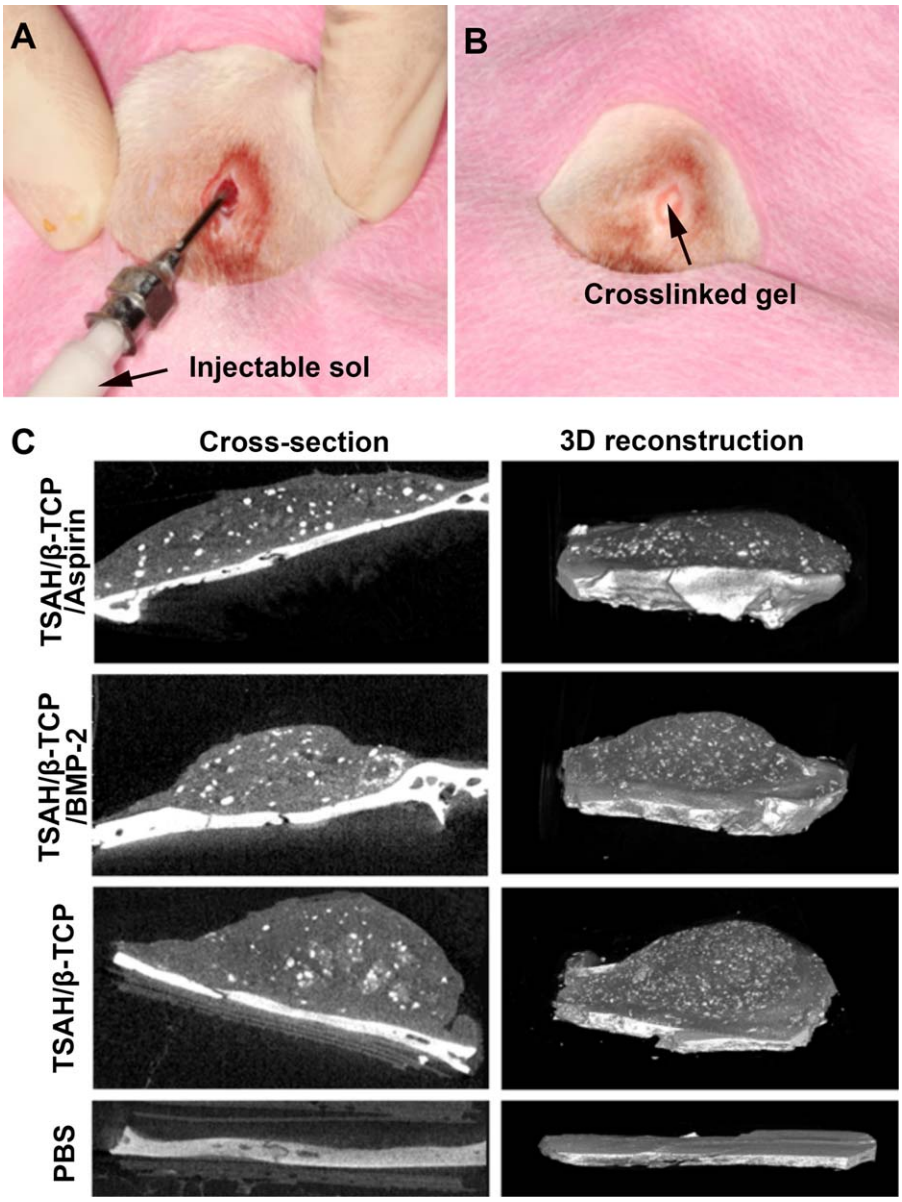

FIGURE 5. An incision was made on the cranial skin of a rat and a subperiosteal tunnelling technique was used to create a space with a needle. One hundred microliter hydrogel solution was injected into the space created (A). After the solution became a crosslinked hydrogel, the incision was closed and sutured (B). Micro-CT images showed successful bone augmentation in all the experimental groups. The TSAH/β-TCP maintained the height of regenerated bone. In the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups, more mineralised tissues were observed (C).

areas, containing numerous fibrocytes and mechanocytes. Small blood vessels were often found to be distributed along the collagen fibers. In the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups, the collagen fibers were more compact and orderly. In addition, osteoblasts and osteoid structures were observed in some areas, consistent with the high-density masses shown in the micro-CT images. The normal anatomical structure of intact bone and the periosteum was observed in the PBS control group. The Masson staining images revealed similar results. Many collagen fibers grew into the hydrogel materials from the host bone. Although quantitative comparison among the four groups was not conducted, more collagen fibers could clearly be observed in the TSAH/β-TCP/aspirin and TSAH/β-TCP/BMP-2 groups than in the group injected with TSAH/β-TCP only. Many erythrocytes were seen in the TSAH/β-TCP/aspirin group (Fig. 8). Denser and deeper blue collagen fibers were seen in the bone part (PBS group, only bone tissue), which suggested that the collagen fibers were mineralized.

In all experimental groups, immunohistochemical staining showed considerable expression of OCN in the newly formed bone tissue [Fig. 9(A–F)], which is regarded as the most specific and significant matrix protein of the late stage of osteogenesis.[27] The mineralized tissue was mainly located between the original bone and the periosteum, around the vacuoles left by β-TCP decalcification, and along the collagen

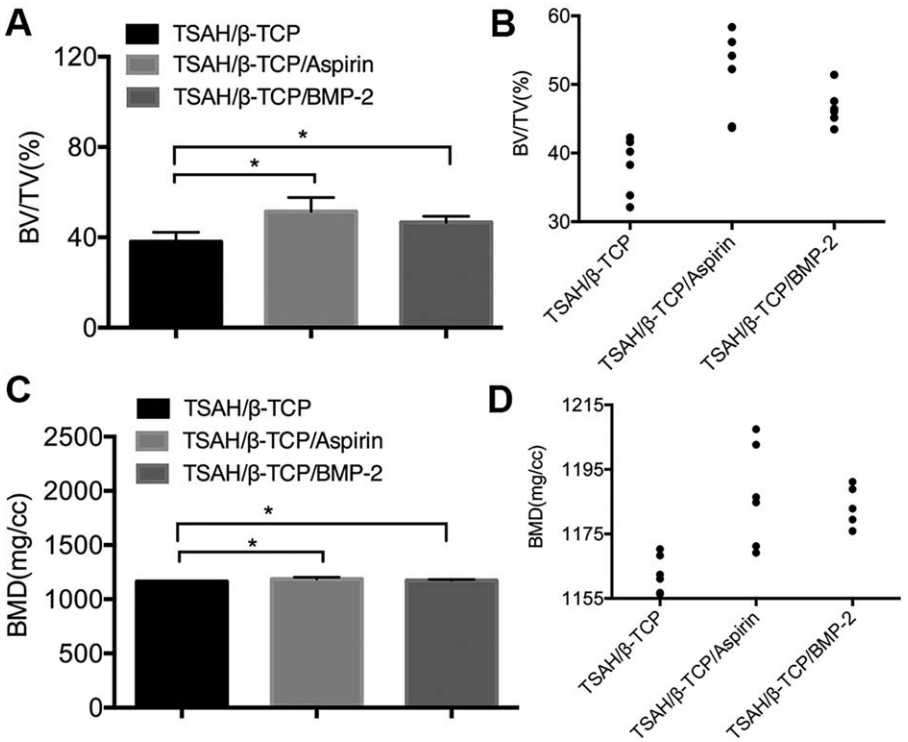

FIGURE 6. The percentage of mineralized tissue (BV/TV) (A) and density of the regenerating tissue (BMD) (B) in the TSAH/β-TCP/BMP-2 group and TSAH/β-TCP/aspirin group were significantly higher than that in the TSAH/β-TCP group, but there is no significant difference between TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin. Scatter plots of BV/TV (C) and BMD (D) show the differences among six rats in each group.

fibers. Mature bone tissue growing from the original bone into the implantation area was detected in the TSAH/β-TCP/BMP-2 group.

Semi-quantitative analysis of OCN staining showed stronger OCN-specific staining tissue in the TSAH/β-TCP/aspirin and TSAH/β-TCP/BMP-2 groups than detected in the TSAH/β-TCP group, with no significant difference between the TSAH/β-TCP/aspirin and TSAH/β-TCP/BMP-2 groups [$p < 0.05$, Fig. 9(M)].

Since vascularization is a coupling key factor of osteogenesis[28,29] the vascularization marker CD34 was also detected, which was mainly expressed at the margin of regenerated bone and along the collagen fibers [Fig. 9(G–L)], with no significant difference among the three experimental groups ($p > 0.05$, Fig. 9(N)]. However, more blood vessels were apparent in the TSAH/β-TCP/BMP-2 group, even in the center of the implantation area.

DISCUSSION

Although injectable bone tissue engineering has attracted increasing attention as a more attractive alternative for minimally invasive bone regeneration, injectable bone tissue materials such as bioceramics, large polymers, and chitosan are associated with various unresolved problems such as poor plasticity, leakage into the surrounding tissues, weak strength and poor degradability, which has thus far restricted their application. Therefore, continued progress is required in this field.[30]

Toward this end, we developed a novel thermo-sensitive hydrogel containing osteoconductive β-TCP particles and the small-molecule drug aspirin, which was shown to promote the early stage of osteogenic differentiation of hMSCs *in vitro* and formation of the late-stage mineralized matrix proteins *in vivo*. The TSAH is a hydrophilic polymer network rich in water, providing an excellent microenvironment for cells to adhere, proliferate, and differentiate;[31] however, it lacks osteoconductivity. β-TCP is known for its excellent biocompatibility, osteoconductivity, and biodegradability, which gradually degrades via dissolution and phagocytosis, creating a calcium- and phosphorous-rich environment, which can promote osteogenesis.[32,33] Thus, the addition of β-TCP particles in the TSAH solution not only maintains the thermo-sensitivity of the TSAH but also confers the material with osteoconductivity. The integration of these two types of materials thus creates a biomimetic matrix composition of natural bone combining the thermo-sensitivity and convenience of the TSAH with the osteoconductivity and biodegradability of β-TCP.

Interconnected pores were widely distributed in the scaffold and the porosity reached 86.78%. Numerous studies have shown that higher porosity could improve osteogenesis, perhaps due to increased ion exchange and osteogenic factor absorption.[34,35] In addition, pores of different sizes favor fluid exchange, cell penetration, and neovascularization. Relatively larger pores favor vascularization and direct osteogenesis, whereas smaller pores promote osteochondral ossification.[36,37]

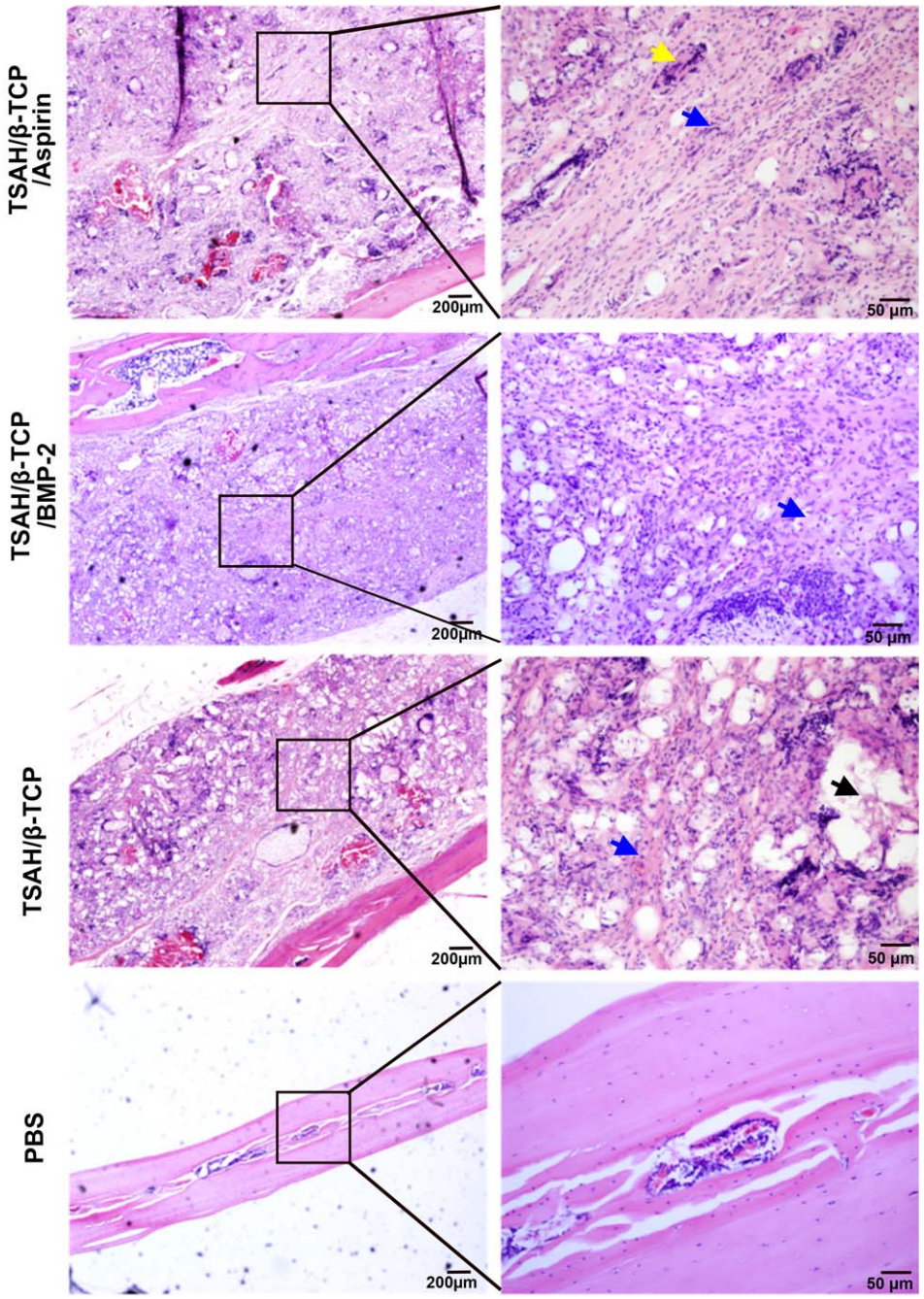

FIGURE 7. HE staining of paraffin sections at week 12 post-surgery. In the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups, the collagen fibers were more compact. Large amount of collagen fibers grew into the implantation areas (blue arrows). Multinucleated giant cells were found adjacent to the remnant TSAH (yellow arrows). The degradation of the hydrogel was observed (black arrows). Osteoblasts and osteoid structures were observed in some areas. Normal anatomical structure of intact bone and the periosteum was seen in the PBS group.

In vitro, aspirin had no effect on the proliferation or viability of hMSCs below 400 μg/mL, in accordance with a previous study showing that aspirin induced cell apoptosis at 400–800 μg/mL.[24] We further showed that a moderate aspirin concentration (100 μg/mL) could enhance the osteogenic differentiation of hMSCs and improve the expression of osteogenic markers of different stages including ALP, Col I, and OCN expression. The viability of hMSCs cultured with 200 μg/mL aspirin showed a tendency to decline. However, the OCN and Col I gene expression of hMSCs cultured with 200 μg/mL aspirin decreased significantly, probably because the osteogenic genes are more sensitive to the concentration of aspirin.

Moreover, the ALP activity of the hMSCs cultured with the supernatant of the aspirin-loaded TSAH/β-TCP was increased, confirming its osteogenic differentiation effect. Furthermore, ALP activity of the hMSCs cultured with the 7th day's supernatant of TSAH/β-TCP/aspirin was higher

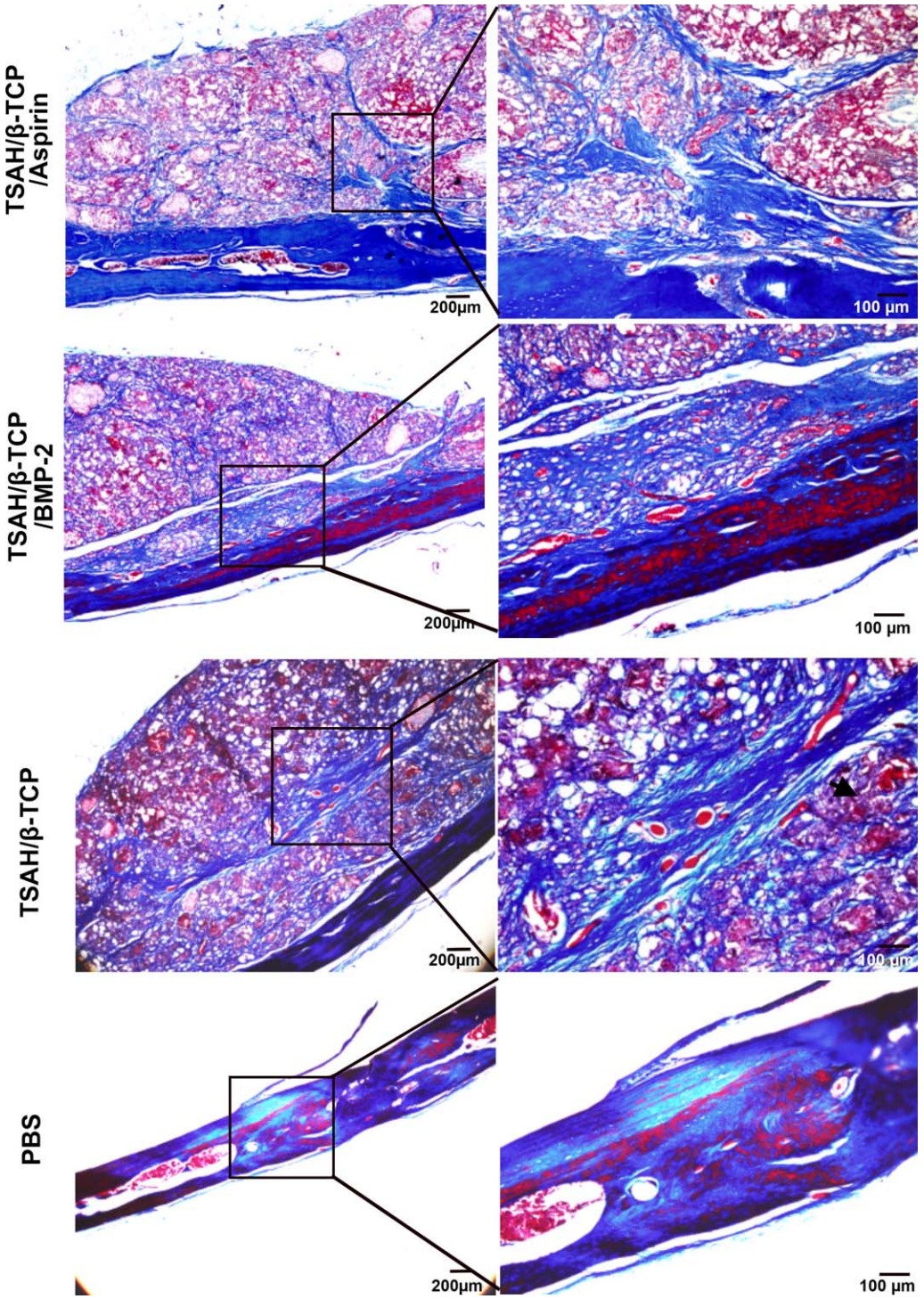

FIGURE 8. Masson trichrome staining of paraffin sections clearly manifested that at week 12 post-surgery, in the TSAH/β-TCP/BMP-2 and TSAH/β-TCP/aspirin groups, the collagen fibers were more compact.

than that cultured with the third day's, which might be due to an increase in calcium and phosphorus ions that dissolved in the supernatant to further promote osteogenesis.

It seems that aspirin alone cannot induce osteogenic differentiation in the absence of Dex *in vitro*. Dex has been routinely used as a classical inducer for osteoblast differentiation *in vitro*.[38–40] The probable mechanism is that Dex induces Wnt/β-catenin signaling-dependent Runx2 expression and enhances Runx2 activity by upregulation of TAZ and MKP1.[38] Aspirin is more like a regulator of bone metabolism. Previous studies have shown that aspirin at a proper concentration could promote osteoblast differentiation via several possible pathways such as the 15d-PGJ2/PPARγ/TGF-β1 [24], Wnt/β-catenin,[21] IFN-γ, and TNF-α[23] pathways

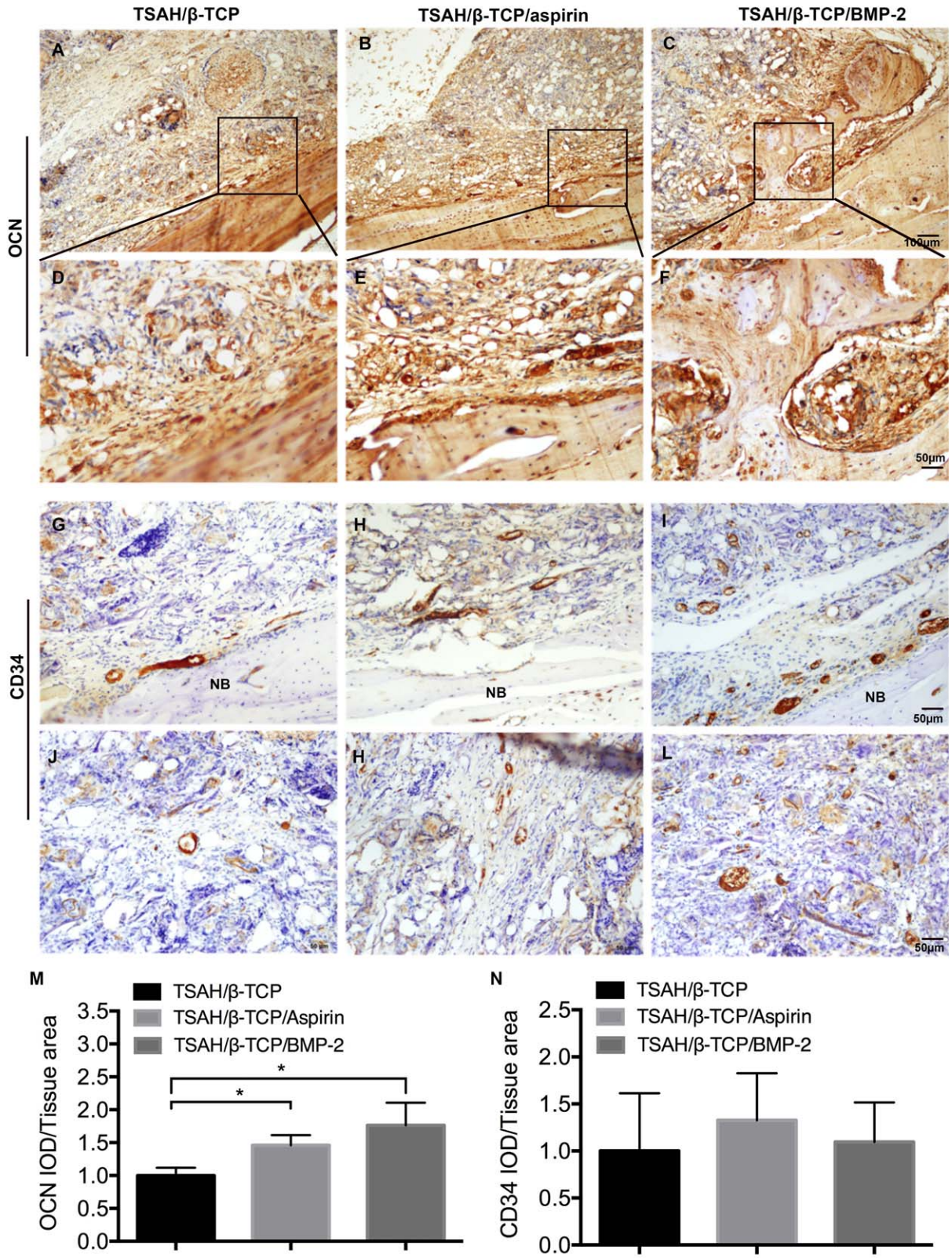

FIGURE 9. In all the experimental groups, immunohistochemical staining showed a considerable expression of OCN in newly formed tissue (A–F). In the TSAH/β-TCP/BMP-2 group, mature bone tissue was observed (C,F). The immunohistochemistry staining of CD34 indicates the newly formed blood vessels in all the experimental groups (G–L). Semi-quantitative analysis showed stronger OCN-specific staining tissue in the TSAH/β-TCP/aspirin and TSAH/β-TCP/BMP-2 groups than in the TSAH/β-TCP group, with no significant difference between the TSAH/β-TCP/aspirin and TSAH/β-TCP/BMP-2 groups (M). No statistically significant difference was found among the three experimental groups in CD34-specific staining (N).

and prevent the formation of osteoclast through inhibition of NFκB pathway.[41,42] In summary, Dex could induce osteogenic differentiation while aspirin could regulate and enhance osteogenesis. The combination of Dex and aspirin results in a synergistic effect. In this study, we did not examine the specific pathway triggering aspirin's osteogenic effect on hMSCs, which will be investigated in our future research.

The release of aspirin showed an initial burst from TSAH/β-TCP and then gradually decreased. To further control and prolong the release, the encapsulation of the drugs into a controlled-release carrier may be an alternative strategy to enhance the osteogenesis ability, which would allow for the gradual release of aspirin from degradable carriers throughout the degradation process.

With the minimally invasive tunneling technique, TSAH/β-TCP was easily injected and *in situ*-gelled under the periosteum of the cranial bone in the rat model. The fluidity of the hydrogel would be a great asset for surgeons. After 12 weeks, micro-CT analysis showed that all of the hydrogel groups had a high-density mineralized matrix in the augmented areas. This suggests that TSAH/β-TCP itself can induce mineralization to a certain degree, which further increased in combination with BMP-2 or aspirin, with no significant difference between the two.

OCN is expressed by mature osteoblasts in the late stages of osteogenic differentiation,[27] and is considered to be the most specific marker of osteogenesis. Immunohistochemical staining showed considerable expression of OCN in the newly formed bone, suggesting that the hydrogel induced bone formation. Furthermore, specific staining around the vacuoles left by β-TCP proved that β-TCP could promote the mineralization of osteoid tissue. Moreover, the addition of either BMP-2 or aspirin increased OCN expression, in accordance with the micro-CT findings. In addition to osteogenesis, we also studied the angiogenesis within the newly formed tissue as functional blood vessels plays a significant role in bone regeneration 28, 29]. Our results showed induction of a high density of blood vessels in all three experimental groups.

BMP-2 is one of the most promising osteoinductive growth factors for bone regeneration but is expensive, whereas aspirin is a very common NSAID. Given the comparable effects to BMP-2, our results suggest that aspirin has promising potential to promote osteogenesis.

The main limitations of the present study are that we did not directly seed hMSCs on the hydrogel to study the direct effects of aspirin-loaded TSAH/β-TCP on osteogenesis and angiogenesis. We also did not study the effect of different concentrations of aspirin on bone augmentation *in vivo*. Therefore, more experiments will be performed to investigate these issues in our future studies.

CONCLUSION

The results from this study show that aspirin-laden TSAH/β-TCP hydrogel has promising potential to promote bone augmentation. The study provides good support for continuing to investigate the potential of the aspirin-laden TSAH/β-TCP hydrogel, and its application in the regeneration of massive and irregular craniofacial bone defects.

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