Synthesis of a Novel bFGF/nHAP/COL Bone Tissue Engineering Scaffold for Mandibular Defect Regeneration in a Rabbit Model

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Abstract: In bone tissue engineering, scaffold fabrication and biocompatibility are crucial concerns. Many scaffold materials have been explored, among which nanometer hydroxyapatite (nHAP) and collagen (COL) are commonly used. Additionally, growth factors can be used to modify scaffolds. In this study, lyophilization technology was used to build a scaffold comprising basic fibroblast growth factor (bFGF), nHAP and COL for the first time. The resulting scaffold was characterized. bFGF release from the scaffold was assessed by ELISA. Bone marrow mesenchymal stem cells (BMSCs) were prepared and seeded onto the scaffold to test in vitro biological compatibility. A scanning electron microscope was used to observe the scaffold and evaluate BMSC morphology, and the cells were counted to detect early cell adhesion. Cell proliferation and activity were assessed by a cell counting kit-8 assay and measurement of alkaline phosphatase activity, respectively. Bilateral mandibular defects were prepared in 12 New Zealand rabbits and repaired using scaffolds. The rabbits were divided into four groups: a group treated with allogeneic BMSC-seeded bFGF/nHAP/COL scaffold, a group treated with allogeneic BMSC-seeded nHAP/COL scaffold, a group treated with nHAP/COL scaffold alone, and an untreated control group. After 12 weeks, three-dimensional computerized tomography examination, computerized tomography value measurement, gross observation and hematoxylin and eosin stain staining were conducted. SPSS17.0 software was used for data analysis. The gross morphology conformed to the characteristics of a tissue engineering scaffold. The bFGF/nHAP/COL scaffold promoted BMSC adhesion, proliferation and differentiation and hence promoted good bone formation, without exhibiting biological toxicity. Our findings show that the bFGF/nHAP/COL scaffold has good physical properties and biocompatibility in vitro, and can be used to promote osteogenesis after in vivo implantation.

Key words: Tissue engineering, Bone defect, nHAP, COL, bFGF

Introduction

Bone is an organ with a complex cellular composition, and bone tissue, with its specialized organic and inorganic architecture, can be defined as microscopic nanocomposite tissue1. Bone defect repair involves a series of complex physiological and pathological processes2. Comminuted fractures, bone tumors, extractions and infections often result in local loss of bone tissue in varying degrees2. Bone grafts and prosthesis implantation are used for bone defect repair and reconstruction, but the results are often unsatisfactory. Acellular allogeneic bone bypasses this limitation, but complications such as nonunion, infection, and stress fractures often occur3.

Bone tissue engineering emerged almost thirty years ago as an alternative approach to repairing bone defects. Since then, this field has received increasing attention and techniques have substantially improved4. In general, an engineered tissue consists of a scaffold, cells, and essential growth factors5-8. Scaffolds with high porosity promote bone regeneration and vascularization9, therefore, the selection of materials and construction of scaffolds are very important considerations. Collagen, a biologically inducible, biodegradable substance with ideal mechanical properties is a major extracellular matrix component that is widely used in structural reconstruction and promotes cell growth and differentiation10. At the same time, nano-scale biomaterials have attracted wide attention in the field because of their good biological and biomechanical properties11. Among various nanomaterials, nanometer hydroxyapatite (nHAP) has been investigated for use mainly because of its potential biocompatibility and osteoinductive properties. nHAP is essentially the same as natural hydroxyapatite, so it has become a common material for bone regeneration and reshaping applications, and dental restoration, partly because it can enhance cell attachment, proliferation, and mineral formation12-14.

Basic fibroblast growth factor (bFGF), a member of the fibroblast growth factor family, has a variety of biological regulatory functions, including neuroprotection, inducing vasodilation, stimulating angiogenesis and inhibiting apoptosis14-16. Wang Lei et al. previously confirmed that bFGF (50 ng/ml) promoted BMSC proliferation17. In the present study, lyophilization was used to mix bFGF with nHAP and collagen (COL) to form a composite scaffold (bFGF/nHAP/COL). Vilquin et al. previously elucidated that bone marrow mesenchymal stem cells (BMSCs) are ideal for use in tissue engineering because they exhibit no immunogenicity18. Early experiments demonstrated that the critical diameter of round, full-thickness bone defects in rabbits is 8 mm19.

In this examination, a composite scaffold was constructed and seeded with cells, and was tested for biocompatibility in vivo and in vitro to identify an ideal scaffold for bone tissue engineering.
Materials and Methods

Materials

Rat tail tendon type I collagen was purchased from Shengyou Biotechnology (Hangzhou, Zhejiang, China). nHAP was purchased from Emperor Nano Material (Nanjing, Jiangsu, China). Acetic acid, anhydrous ethanol, hydrochloric acid (HCl), and cell counting kit-8 (CCK-8) and alkaline phosphatase (ALP) kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Phosphate buffer solution (PBS), alpha minimum essential medium (αMEM), 10% fetal bovine serum (FBS), and 0.25% trypsin were purchased from GE Healthcare Life Sciences Hyclone Laboratory (South Logan, Utah, USA). bFGF was purchased from PeproTech (Rocky Hill, New Jersey, USA). bFGF ELISA kits were purchased from the Yuduo Biotechnology Company (Shanghai, China). All other general laboratory reagents were of the highest quality available and were obtained through standard commercial suppliers.

Preparation of bFGF/nHAP/COL scaffolds

Acetic acid was diluted to 0.005 mol/L in deionized water. COL (10 mg) was added to 10 ml of the acetic acid solution and stirred with a JB-2A stirrer (Bante Instruments Limited, Shanghai, China) for 50 minutes. nHAP (10 mg) was then added and the solution was stirred overnight. The quality ratio of the solution yielded by the above method (nHAP:COL) was 1:1.5. bFGF was dissolved in PeproTech protein solution to 5 ng/μl(20) and then added to the scaffold solution and stirred at a low temperature for 50 minutes, so that the final concentration of bFGF in the mixed solution was 50 ng/ml. The solution was then transferred to a 24-well Teflon culture plate and frozen at −20°C for 24 h, and was then lyophilized at −80°C for 48 h using a VFD-2000 (Boyikang, China) to obtain bFGF/nHAP/COL scaffolds. The morphology and microstructure of the scaffolds were observed with a scanning electron microscope (SEM)(S-4800, Hitachi Co., Tokyo, Japan).

Detection of scaffold porosity, swelling capacity, water absorption capacity, mechanical properties and degradability

Scaffolds were cut into an appropriate shape. The volume was recorded as Vs and the quality as ms. The density was calculated as ρs = ms / Vs. Each sample was measured three times and the average value was recorded. Scaffolds were immersed in anhydrous ethanol for 24 h and then dried and weighed. The quality was recorded as m. The volume of the inner hole was recorded as Vp: Vp = (m − ms) / ρs. The porosity of scaffold was calculated as Vp / Vs. Each sample was measured three times and the average value was recorded(21).

Sample volume was recorded as V1, and then each sample was immersed in deionized water at room temperature for 24 h. The wet volume was recorded as V2. The swelling capacity was calculated as (V2 / V1 − 1) × 100%(22). After excess water was wiped off, the wet weight was recorded as S1. The scaffold was dried in an oven (Yuejin Medical Optical Instruments, Shanghai, China), and the dry weight was recorded as S2. The water absorption capacity was calculated as (S2 / S1 − 1) × 100% (22,23).

The compression strength and modulus were obtained by calculation of a stress–strain curve. A micro-load fatigue testing machine (MMT-101NN-10, SSM, Tokyo, Japan) was used at room temperature. The load was 100 N, and the speed was 0.5 mm/min. The diameter of each sample was 13.73 mm, and the height was 10.00 mm. Three samples were tested to obtain an average compression strength and modulus and the standard error was calculated. The compression modulus was defined as the slope of the initial linear part of the stress–strain curve. From 1% strain, a straight line was drawn parallel to the initial linear section of the curve, and the ordinate of the intersection with the stress–strain curve was taken as the compression strength(24).

The initial quality of each sample was recorded as m0, then the samples were placed in a 24-well culture plate and sterilized using a Co-60S(Q(H)630 (Shanqiang Nuclear Radiation Engineering Technology, Beijing, China). The wells were filled with PBS, then the plates were sealed with sterile tape and placed in a cell culture incubator (Heng Scientific Instrument, Shanghai, China) at a constant temperature of 37°C. The samples were washed with deionized water and analyzed at 1, 7, 14, 28, and 56 days(25). The four samples were measured, and the pH of the degradation liquid was determined. The samples were removed from the PBS, washed and dried, and lyophilized for 48 h. The dry weight was recorded as m1. The degradation rate was calculated as (m0 − m1) / m0.

Detection of bFGF release from scaffolds

Each standard scaffold was fabricated from 1 ml bFGF/nHAP/COL (containing 50 ng bFGF). Three scaffolds were placed into wells of a plate and 1 ml of 1× PBS buffer (pH 7.4) was added to the wells. The plates were sealed with a membrane and placed in a cell culture incubator at 37°C. PBS was collected from the pores of the samples at specific time points (1, 2 to 20 days). PBS(1 ml) was added to the collected liquid and a bFGF ELISA kit (Yuduo Biotechnology, Shanghai, China) was used to detect the bFGF content of each sample. The mean bFGF content was determined three times and cumulative bFGF release was calculated and plotted(26).

Preparation and identification of BMSCs

The identity of rabbit BMSCs (BioHermes, Wuxi, China) was confirmed by inducing osteogenesis. β-glycerophosphate (10 mmol/L), dexamethasone (10 nmol/L) and ascorbic acid (50 g/ml) were added to αMEM containing 10% FBS to produce an osteogenic medium. Third-passage BMSCs were digested with trypsin. The digestion was terminated by addition of αMEM containing 10% FBS, then this mixture was centrifuged. The supernatant was discarded and the cells were resuspended in fresh medium. The cell suspension was adjusted to 2 × 10^5 cells/ml, and then 1 ml of cell suspension was seeded per well in 24-well plates. αMEM containing 10% FBS was added to the wells and the cells were cultured at 37°C with 5% CO2. When the BMSCs reached approximately 80% confluence, the culture medium was discarded and the cells were washed three times. Osteogenic media was then added and the cells were cultured at 37°C with 5% CO2. The culture medium was changed every other day. On day 21 after the start of osteogenic induction, the cells were fixed with 4% paraformaldehyde. Calcium nodules were stained with 0.2% alizarin red and observed using a microscope(CKX41, Olympus, Tokyo, Japan)(27).

Cell culture

The scaffolds were disinfected, then were immersed in Dulbecco’s modified Eagle’s medium containing 10% FBS for 24 h. Suspended BMSCs were adjusted to 1 × 10^5 cells/ml. 100 μl medium (100 μl) was added into the wells of 24-well plates, and then scaffolds were placed into separate wells. The cell suspension was added to the surface and cultured for 2 h in at 100% humidity.

BMSC morphology

Cell morphology was examined using an inverted light microscope (CKX41,Olympus Co., Tokyo, Japan). After the cells were incubated for 72 hours, the samples were removed, washed with PBS, and fixed with glutaraldehyde solution overnight at 4°C in a humidified...
Surgical procedure and implantation

Protein. ALP activity was recorded as U/g protein. The total amount of protein was measured using the bicinchoninic acid method and ALP activity was normalized to total protein. ALP activity was recorded as U/g protein.

The BMSCs were adjusted to 1 × 104 cells/ml and added to culture plates that were pre-coated with bFGF/nHAP/COL or nHAP/COL scaffolds (1 ml per well). BMSCs in culture plates with no scaffold served as a control group. Six wells were prepared for each group and the cells were cultured at 37°C with 5% CO2. The number of non-adherent cells was counted at 1, 2, 4, 8, and 24 h and the adhesion rate was calculated according to the following formula: adhesion rate (%) = (number of seeded cells − non-adhered cells) / (number of seeded cells) × 10027.

CCK-8 assay

Aqueous soluble tetrazolium/formazan (CCK-8 kit, Dojindo, Maryland, USA), an indicator of NADH- and NADPH-dependent dehydrogenase activity, was used to assess cell proliferation in the two scaffold groups and the control group. At 1, 3, 5, and 7 days after incubation, three samples from each group were assessed. CCK-8 solution (100 μl) was added to each well and the culture plate was incubated at 37°C with 5% CO2 for 4 h. A 300 μl aliquot was removed from each well and transferred to a 96-well culture plate. The absorbance of the solution at 450 nm was measured with a microplate reader (Bio-Rad, California, USA). Differences were considered significant when P < 0.05.

Statistical analysis

All data are expressed as means ± standard deviation. Analysis of variance and the Student’s t-test were used to compare groups. Data analysis was conducted using SPSS 17 statistical software (SPSS, Inc., Chicago, USA). Differences were considered significant when P < 0.05.

Results

bFGF/nHAP/COL scaffold morphology and scanning electron microscopy

There was no apparent difference in morphology between the nHAP/COL and bFGF/nHAP/COL scaffolds, so only the latter is described here. The scaffold was white and exhibited good flexibility, and gradually returned to its original state after compression deformation. The surface was slightly rough (Fig. 1). The three-dimensional structure of the scaffold was porous, and a large number of nHAP particulates were attached to the surface of scaffold.
Table 1. Porosity, pore size, swelling index, water absorption of the bFGF/nHAP/COL scaffolds.

<table>
<thead>
<tr>
<th>Porosity (%)</th>
<th>Pore size (μm)</th>
<th>Water adsorption (%)</th>
<th>Swelling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>93.1±6.0</td>
<td>130±29</td>
<td>245±5</td>
<td>33.4±0.9</td>
</tr>
</tbody>
</table>

Results are expressed as the means±SD (n=3).

Table 2. Compressive strength and compressive modulus of the bFGF/nHAP/COL scaffolds.

<table>
<thead>
<tr>
<th>Compressive strength (MPa)</th>
<th>Compressive modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.24±0.92</td>
<td>0.58±0.12</td>
</tr>
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</table>

Results are expressed as the means±SD (n=3).

Figure 3. (A) In the process of degradation, the pH values of bFGF/nHAP/COL scaffold were gradually increased and finally stabilized between 7.3 and 7.4. (B) The changes of degradation rate during the course of degradation. With the degradation time increased, the degradation rate of the bFGF/nHAP/COL scaffolds increased steadily.

Figure 4. Cumulative release rate of bFGF per time point from bFGF/nHAP/COL scaffolds.

Figure 5. (A) Alizarin red S staining verified the formation of mineralized nodules. (B) Alizarin red S staining verified the formation of mineralized nodules.

Porosity, swelling capacity, water absorption capacity, mechanical properties and degradability

Porosity, swelling capacity, water absorption, compressive strength and compressive modulus analysis indicated that the physical properties of the scaffold met the basic requirements for use in tissue engineering (Tables 1 and 2). One hour after the start of the experiment, the pH was approximately 7.0. From days 1 to 7, the pH increased gradually, then it stabilized at between 7.3 and 7.4 after 10 days. The low pH in the early stage resulted from residual acetic acid in the COL solution. As this acetic acid evaporated, the pH tended to rise and achieved the desired physiological value (Fig. 3A).

Samples were removed from the PBS, dried, and freeze-dried, then examined for degradation. At 1 h after the start of the experiment, the degradation rate was close to 0. After a day, the degradation rate showed a gradual increase, but tended to be slow. The degradation rate was 5.06%, 8.93%, 12.10%, 14.65%, 18.47% and 21.18% at 1, 2, 3, 4, 6 and 8 weeks after the start of the experiment (Fig. 3B).

bFGF release from scaffolds

bFGF release from the bFGF/nHAP/COL scaffolds occurred for 17 days. By day 18, the amount of bFGF released into the supernatant by each sample was small (< 1 ng) or undetectable. The total amount of bFGF released by the scaffold was 91.05 ± 3.38%. Between days 3 and 5, the amount of bFGF released from the scaffolds suddenly increased, and then the release continued more gradually after day 5 (Fig. 4).

Calcified nodules confirmed the identity of BMSCs

After osteogenesis was induced in third-passage BMSCs for 7 to 14
89 days, microscopy revealed crystalline masses of different sizes. These were calcified nodules that formed early in the differentiation process. The proliferation rate of BMSCs obviously decreased over time. Over the course of induction, the number and area of the crystalline masses increased, and dark masses were visible, which were calcified nodules. A small number of nodules were removed and floated in the medium during the change of culture medium. After induction, the cells were fixed with paraformaldehyde and stained with alizarin red S, and red-stained calcified nodules were visible (Fig. 5 and B).

Cell morphology

Cell morphology was observed using an inverted light microscope (CKX41; Olympus). The BMSCs exhibited high survival rates and rapid proliferation. During 6 days of culture, the cells proliferated vigorously, and showed a high density in the early stage and no obvious deformation in passage. Images acquired with an SEM showed cells randomly distributed over the surface and interior of the scaffold. After 3 days of culture with the scaffold, a large number of cells adhered to the scaffold surface and pores. The cell aggregation range was enlarged and the proliferation phenomenon was active (Fig. 6A). Observations with an SEM showed that the single cells displayed polygonal or triangular morphology, and microfilaments were tightly attached to the scaffold (Fig. 6B).
bFGF promotes BMSC adhesion

The cell adhesion rate was enhanced by increasing the incubation period in three groups. Following culture for 1, 3, and 6 h, the adhesion rate of the scaffold groups was higher than that in the control group (P < 0.05), and the adhesion rate in the bFGF/nHAP/COL group was higher than that in the nHAP/COL group (P < 0.05). Significantly more cells had adhered after 3 and 6 h than after 1 h (P < 0.05). These results demonstrate that bFGF increased BMSC adhesion (Fig. 7).

BMSC proliferation is altered by bFGF

BMSC proliferation in the bFGF/nHAP/COL, nHAP/COL and control groups was compared after 1, 3, 5 and 7 days of culture using a formazan production assay. The absorbance values for the scaffold groups increased, indicating significant cell growth within the scaffolds. The number of cells increased with culture duration in all three groups. BMSC proliferation was observed in all three groups, and there was no significant difference between the scaffold groups and the control group after 1 day (P > 0.05). However, after 3 days, the number of cells in the scaffold groups was higher than that in the control group (P < 0.05). Moreover, there was a significant difference between the bFGF/nHAP/COL and nHAP/COL groups (P < 0.05). These results demonstrate that bFGF promoted BMSC proliferation (Fig. 8).

bFGF promotes ALP activity in BMSCs

No significant differences in ALP activity were identified between the scaffold groups and the control group during the first 4 days (P > 0.05). However, ALP activity in the scaffold groups was significantly increased compared with that in the control group between days 4 and 10 (P < 0.05). Moreover, ALP activity levels were higher in the bFGF/nHAP/COL group than in the nHAP/COL group (P < 0.05). This suggests that the nHAP/COL scaffold effectively promoted BMSC activity and that bFGF further enhanced this impact. Additionally, the rate of increase in ALP activity in all three groups increased rapidly with increasing culture duration, until the values peaked after 10 days (Fig. 9).

Animal and clinical observations

Mandibular defects were created in twelve rabbits. All of the rabbits exhibited postoperative swelling in the week following the surgery, but no functional consequences of the surgery were observed. No rabbits developed abscesses or had to be sacrificed. Samples were taken 12 weeks after surgery (Fig. 10A, B).

Radiographic analysis

To observe new bone formation and bone defect repair, 3D-CT and X-ray images were acquired and CT values were analyzed 12 weeks postoperatively. This analysis confirmed that more new bone was generated in the BMSC/bFGF/nHAP/COL group than in the other groups. A large amount of granulation tissue without new bone was generated in the BMSC/bFGF/nHAP/COL group than in the other groups. (Fig. 12)

Gross observation of specimens

After 12 weeks, the rabbits were sacrificed and the mandible was observed. New bone formation in the defect area was most evident in the BMSC/bFGF/nHAP/COL group, compared with the other groups. No new bone formed in the control group; rather, the defect was completely filled with granulation tissue (Fig. 13).

Histological analysis

The BMSC/bFGF/nHAP/COL group exhibited the greatest osteogenic effect at 12 weeks, evident as new bone formation in the implanted area, compared with other cells/scaffold or scaffold only groups, and with the control group, in which no new bone was visible. Osteoid matrix formed near the junction of the host bone and the implant, and also formed to a similar extent inside the scaffold and adjacent to the bone wall (Fig. 14).

Discussion

Lyophilization technology was first used in food processing. It ensures that the biological activity of proteins is not changed or lost; therefore, it has been gradually applied in the field of medicine. Wang et al. demonstrated minimal loss of unstable proteins during
In tissue engineering, there are many ways to control the release of factors, for example use of nanoparticles, hydrogels, or porous scaffolds. Jun et al. effectively achieved controlled release of growth factors using tissue engineering scaffolds created by lyophilization. It also demonstrated that lyophilization can produce a controlled release effect. Collagen exhibits good adsorption of protein factors. Therefore, lyophilized, porous, spongy collagen scaffolds ensure controlled release of growth factors. During the lyophilization process, the component molecules produce a stronger polymerization force, thus ensuring maintenance of degradation, and improving the physical performance of the scaffold to a certain extent.

nHAP has good biocompatibility and has been widely used in artificial clinical materials. Its addition increases the surface area of a scaffold, thus increasing the adhesion area. Therefore, an nHAP/COL scaffold adsorbs bFGF very well and exhibits good controlled release. In the present experiment, bFGF release was sustained over almost 20 days. The traditional form of bone defect regeneration comprises four stages: hematoma formation, hematoma organization, bone callus formation and callus remodeling, and the critical period is the first 3 weeks. Therefore, the bFGF release duration achieved in this study essentially meets the physiological requirements for bone defect regeneration. CCK-8 and ALP tests showed that bFGF promoted BMSC proliferation and differentiation. Scaffolds without bFGF also promoted proliferation and differentiation to a lesser extent, thereby demonstrating the non-toxic nature of the scaffold.

The biocompatibility of the bFGF/nHAP/COL scaffold has been well documented in vitro, and we further studied this in vivo. When a bone defect reaches a certain range, no new bone forms through the normal physiological healing process; this is considered a critical bone defect. The practical significance of this to bone tissue engineering is that when a critical bone defect occurs, new bone can be induced by introduction of a biological scaffold seeded with cells and loaded with growth factors to regenerate the bone defect. During bone formation, the scaffold is degraded continuously, and the product is not biologically toxic. Allogeneic BMSCs, which are not immunogenic, are ideal seed cells.

In this study, during the preparation of critical bone defects in rabbit mandibles, the periosteum on both the buccal and lingual sides was removed, thus preventing periosteum osteogenesis. No new bone formation was observed in the control group; rather, a large amount of granulation tissue formed, which demonstrated that a critical bone defect had indeed been created. A certain amount of bone formation was observed in the defect in the nHAP/COL group because scaffold itself provided a space for autogenous BMSCs to accumulate, and nHAP and COL exhibited some inductive effect. The addition of allogeneic cells in the BMSC/nHAP/COL scaffold group increased the chance of osteoblast induction, and thus the osteogenic effect was further enhanced. bFGF plays an important role in promoting cell proliferation and differentiation, thus indirectly promoting new bone formation. Therefore, in the BMSC/bFGF/nHAP/COL group, all of
our results confirmed an obvious osteogenic effect. Taken together, our results indicate that the bFGF/nHAP/COL scaffold has good physical properties and biocompatibility in vitro, and promotes osteogenesis in vivo.

Ethical approval and consent to participate
In the experiment, 12 healthy New Zealand rabbits (both male and female, weighing about 2.5 kg) were obtained from the experimental animal center (Shenyang, China) of China Medical University. Animal license No. scxk-In2011-0009, animal use agreement No. cmu62043006. All experiments and surgical procedures were approved by the China Medical University Committee on animal care and use, and were followed by the National Institutes of Health’s laboratory animal care and use guidelines. All efforts were made to minimize the number of animals used and their suffering.

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Conflict of interests
The authors have no COI existed.

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