Scaffolds of bioactive glass (Bioglass®) combined with recombinant human bone morphogenetic protein-9 (rhBMP-9) for tooth extraction site preservation

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ABSTRACT

Objective: The study aimed to investigate the osteogenic ability of bioactive glass (bioglass) combined with recombinant human bone morphogenetic protein-9 (rhBMP-9) on rat bone marrow mesenchymal stem cells (BMSCs) in vitro. The study also compares bone regeneration using rhBMP9 soaked with different carrier systems, including bioglass or collagen membranes (BioGide, BG) in a rat alveolar bone site preservation model in vivo.

Methods: Scanning electron microscopy was employed to analyze bioglass surface. The absorption and release potential of rhBMP9 from bioglass were researched by ELISA. The cell viability, adhesion, proliferation, and differentiation were assessed for rhBMP9 soaked on bioglass by cck-8 kit, alkaline phosphatase (ALP) activity assay, alizarin red staining, and real-time PCR. Furthermore, prepared grafts (bioglass + BG, bioglass/rhBMP9 + BG, and bioglass + BG/rhBMP9) were implanted into the maxillary right first incisor sockets of Sprague Dawley rats for 8 weeks, and new bone formation was quantified by micro-CT and histological analysis.

Results: Bioglass absorbed rhBMP9 dramatically and released it with a slow and stable speed within ten days by ELISA. When used with cck-8 kit detection, cell viability at 24 h, cell adhesion rate at 8 h, and cell proliferation at 1, 3, and 5 days were decreased in the bioglass alone group versus the control group but slightly increased with the addition of rhBMP9. Similarly, the effect of osteogenic differentiation on bioglass increased significantly when combined with rhBMP9 by upregulating the expression of ALP, mineralized matrix, and osteogenic related genes. Furthermore, both bioglass/rhBMP9 + BG samples and bioglass + BG/rhBMP9 samples significantly improved several bone formation parameters compared with bioglass + BG samples. Interestingly, bioglass + BG/rhBMP9 samples demonstrated more bone regeneration in rat site preservation models.

Conclusions: Both bioglass and BG can be applied in GBR surgery as effective carriers of rhBMP9. However, BG may be more suitable than bioglass for investigating site preservation effect after tooth extraction when associated with rhBMP9 and provides a practical clinical solution to the problem of bone deficiency caused by alveolar bone atrophy.

1. Introduction

With improvements in aesthetic and living standards, oral implant restoration technology has been widely used in clinics due to its good applicability and aesthetic effect [1]. However, implant restoration has higher requirements depending on bone tissue conditions. After conventional tooth extraction, the alveolar bone undergoes progressive resorption and remodeling in the process of physiological healing, which seriously affects the stability of the implant, aesthetic effect, and recovery of masticatory function [2]. In recent years, alveolar ridge preservation technology has exhibited the capacity of maximum retention of the shape and volume of soft and hard tissues by implanting bone or bony substitute materials at the implanted sites [3]. Several animal and clinical trials have previously shown that site preservation can slow resorption rate and better retain the alveolar bone [4, 5, 6, 7].

Bone tissue engineering utilizes tissue engineering principles and methods to repair bony defects with cells or cytokines combined with a 3D biological scaffold [8]. The integration of growth factors with biomaterials was clinically recommended as the standard strategy for bone regeneration, enhancing the therapeutic effect in implant surgery [9, 10].

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The ideal bone repair materials typically present excellent biocompatibility and porous structure conducive to cell adhesion and nutrient transport [11]. Among the autografts, allografts and xenografts belong to traditional bone grafts; autografts are considered the gold standard for repairing bony defects but are limited by the bone quantity, severe trauma, and easy infection of donor bone [12]. Allografts and xenografts present a risk of immune rejection and potential disease spread [13]. Recently, attention has been shifted to synthetic biomaterials. Bioactive glass can form a hydroxyapatite layer, similar to host tissue, through the interface facilitated in establishing a microenvironment for bone conduction and stimulation [14, 15, 16]. The abilities of bioglass in promoting osteoblast differentiation and bone matrix mineralization were improved [17]. Yuan [18] confirmed that bioglass has osteoinductive activity in animal soft tissues. Ghosh [19] also discovered that bioglass had faster angiogenesis and stronger interface strength on comparing bioglass with traditional bioceramics for repairing lateral radius defects in goats.

At present, the growing use of growth factors has become a current trend in bone tissue engineering, such as FGF, TGF-β, IGF, PDGF, VEGF, and BMP [20, 21, 22, 23]. Bone morphogenetic proteins (BMP) are highly conserved secretory multifunctional proteins with similar structures, which are widely recognized due to their positive role in developing skeletal system and reconstruction of bone or cartilage [24]. BMP9 is reported to be the most potent inducer in stimulating osteogenic differentiation and new bone formation [25, 26, 27, 28]. Fujioka-Kobayashi [29, 30], a Japanese scholar, reported that rhBMP9 loaded in different scaffolds could markedly improve the ALP activity of bone stromal cells and the expression level of osteogenic differentiation genes. A large number of animal experiments have also verified this conclusion by combining rhBMP9 with various grafts. Nie [31] discovered that recombinant adenovirus-mediated BMP9 transfection into rDFCs seeded in CHA could significantly repair periodontal bone loss. In the rat model of a bilateral skull defect, Nakamura [32] confirmed that the collagen sponge with rhBMP9 had a greater area of new bone formation than the collagen sponge alone. In nude mice, BMP9 also showed a strong potency in subcutaneous ectopic osteogenesis with grafts [33, 34, 35]. BMP9 was considered the most optimal choice in bone tissue engineering. However, most studies on the osteogenic potential of BMP9 were through adenovirus transfection, which is not yet approved by the FDA for clinical use. Therefore, the authors considered exploring the possibility of constructing tissue engineering bone by seeding BMP9 on bioglass directly.

Guided bone tissue regeneration (GBR) has become a site preservation method with the longest clinical application time. Researches displayed that rational use of GBR could favor dental implant and presented good osseointegration in 4–6 months, with the excellent long-term effect of restoration [36, 37]. The Bio-Gide collagen membrane (BG) was highlighted as a guided tissue regeneration membrane that provided a stable internal environment for bone tissue repair during GBR surgery [4]. However, in the cases of large bone defects, the osteogenic capacity of GBR alone was unsatisfactory. Some scholars have tried to apply tissue culture plastic (Bioglass®) with rhBMP9 for implant surgery [29]. However, in the cases of large bone defects, the osteogenic capacity of GBR alone was unsatisfactory. Some scholars have tried to apply tissue culture plastic (Bioglass®) with rhBMP9 for implant surgery [29]. Meanwhile, some studies discovered that different carrier systems had different effects on delivering BMP9 in GBR. Moreover, some studies discovered that loading BMP9 into collagen membrane could obtain a more obvious bone augmentation than bone substitutes [39, 40], indicating that collagen membrane may be more suitable to incorporate with BMP9 in GBR models.

Based on the research of bone tissue engineering, the present study aimed to examine the adsorption and release the potential of bioglass to rhBMP9. Viability, attachment, proliferation, and differentiation of BMSCs were investigated in vitro by seeding cells on bioglass with/without rhBMP9 compared to standard tissue culture plastic. Meanwhile, rhBMP9 was implanted into the alveolar socket by GBR technique to evaluate bone regeneration potential of rhBMP9, and the osteogenic activity between different carrier systems was compared.

2. Material and methods

2.1. Cell experiments

RhBMP9 was purchased from Abcam Inc in Cambridge (Massachusetts, US). Bioglass® Synthetic Bone Graft was provided by Public Medical Technology (Shenzhen) Co. Ltd. Rat bone marrow mesenchymal stem cells (BMSCs) were obtained from Zhong Qiao, Xin Zhou Biotechnology (Shanghai) Co. Ltd. For all experiments in vitro, the following three groups were examined: (1) Control; tissue culture plastic (2) Bioglass only (3) Bioglass + rhBMP9 (100 ng/mL).

2.1.1. Scanning electron microscopy images

Bioglass samples were sputter-coated using an ion coater device with 10 nm of gold and analyzed microscopically by a scanning electron microscope, as previously described [29].

2.1.2. rhBMP9 adsorption quantification with ELISA

After a soaking period incubation of 100 ng/mL of rhBMP9 onto bioglass at 37 °C in a shaking incubator, the remaining PBS solution containing unattached protein was collected and quantified by an ELISA Kit for BMP9 (Cloud-Clone Corp Co Ltd, Wuhan). Subtraction of total soaked protein from the amount of unadsorbed protein was used to determine the amount of adsorbed protein to the surface of bioglass as previously described [30]. In addition, when the adsorption amount was >90%, the bioglass was taken out and soaked in a PBS solution of 1 ml. Then samples were collected at various time points, including 15 min, 1 h, 8 h, 24 h, 3 d, and 10 d. All samples were duplicated, and three independent experiments were carried out.

2.1.3. Cell viability, adhesion, and proliferation test

2.1.3.1. Cells culture.

Cells were routinely cultured at 37 °C in a humidified atmosphere of 5% CO₂ incubator in a complete medium comprising DMEM (Gibco, Life Technologies, Carlsbad, CA), 10% fetal bovine serum (Gibco), and antibiotics (Gibco). Then the unattached cells were removed by changing the liquid every 3–4 days to achieve cell culture. At the 3rd passage, cells were resuspended for further research. The morphology and state of cells were observed and recorded under an inverted phase-contrast microscope daily.

2.1.3.2. Cell viability, adhesion, and proliferation observation.

Bioglass was placed at the bottom of 24 well plates and soaked with rhBMP9 in DMEM for 5 min. After that, cells were seeded at a density of 12500/cm² per well for cell viability, adhesion, and proliferation experiments. After one day of culture, cell viability was observed by fluorescent microscope. Adhesion and proliferation were detected using Cell Counting Kit-8 assay (CCK-8, Solarbio, Life Sciences, Beijing) at 8 h, 1d, and 5d.

2.1.4. Real-time PCR analysis for osteoblast differentiation markers

On the 3rd and 14th day, the total mRNA of each group was extracted by using RNA Extraction Trizol (TIANGEN, China) and reverse transcription into cDNA by MonScript™ RTII (Monad, China) according to the manufacturer's instructions. Then the mRNA expression of the osteogenic gene (Runx2, ALP, OCN) according to Table 1 in different groups was detected by Real Time-qPCR. All samples were run in triplicate and normalized by the expression of GAPDH. The primers were synthesized by Sangon Biotech. The detailed primer sequence information was as follows:
rhBMP9 was then used to study the Japanese scholar Fujioka-Kobayashi [29, 30, 40, 42, 43, 44]. Then, a series of studies on rhBMP9 compounds and various carrier materials by saline (control), in which the amount of rhBMP9 was taken based on a standard experiment. To stop bleeding, a central incisor. Sterilized gauze was used to suppress the extraction fossa carefully separated using a dental probe. The central incisor was loosed, and the extraction fossa filled with materials immediately according to the protocol. On the 7th day after culture, the chromogenic substrate solution and paraformaldehyde for further research. It was observed that the adsorption rate of rhBMP9 was close to 90% after pre-soaking and then released slowly. From 15 min after soaking to the end, the function of osteogenic induction of MSCs; its activity can directly reflect the functional state of osteoblasts.

### 2.1.5. ALP activity assay

On the 7th day after culture, the activity of ALP in the supernatant was surveyed. Each assay condition was performed in triplicate, and the results were repeated in at least three independent experiments. ALP is an early marker in the process of osteogenic induction of MSCs; its activity can directly reflect the functional state of osteoblasts.

### 2.1.6. Mineralization assay

On the 14th day after culture, the extracellular mineralized matrix was defined by an alizarin red staining kit (Solarbio, Life Sciences, Beijing). Cells were fixed in 96% ethanol for 15 min at room temperature, stained with 0.2% alizarin red solution for 1 h, and the mineralized matrix was observed under a bright-field microscope (Leica optical microscope, Germany).

### 2.2. Animal experiments

Twenty male Sprague Dawley rats were purchased from Beijing Hua-fukang Company (aged 8 weeks and weighing 190–210 g), were distributed into four separate cages with a standard diet and water. The room temperature was maintained at 22–24 °C and synchronized for a light-dark cycle of 12 h. All protocols were carried out as per the Ethical Guidelines of the Animal Protection Association and were approved by Animal Care and Ethics Committee. To compare the osteogenic potential of rhBMP9 soaked on different carrier systems, including synthetic bio-materials (Bioglass) or collagen barrier membranes (BioGuide) in vivo and a more reliable scaffold material for transmitting rhBMP9, rat alveolar bone site preservation models were prepared as described previously [41].

### 2.2.1. Surgical procedures

After intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg), each SD rat was disinfected by iodophor in the maxillary central incisor area. Gingiva around the right maxillary central incisor was carefully separated using a dental probe. The central incisor was loosened, and the alveolar bone defect was created by completely extracting the central incisor. Sterilized gauze was used to suppress the extraction fossa to stop bleeding, then filled with materials immediately according to the following groups (i) control (empty) (n = 5), (ii) Bioglass + BG (Bioguide, 0.5 mm in thickness, Geistlich Pharma AG) (n = 5), (iii) Bioglass/2 μg rhBMP9 + BG (n = 5), (iv) Bioglass + BG/2 μg rhBMP9 (n = 5). Either BG (cut in 2 mm diameter circle) or 2 mg of Bioglass were implanted directly in the incisive fossa and soaked with 2 μL of rhBMP9 solution or sterile saline (control), in which the amount of rhBMP9 was taken based on a series of studies on rhBMP9 compounds and various carrier materials by the Japanese scholar Fujisawa and colleagues [29, 30, 42, 43, 44]. Then, the surrounding mucosal tissue was pulled up and sutured. Six weeks after the procedure, rats were sacrificed, and maxillary alveolar bone tissue on the extraction side was dissected carefully and fixed in 4% paraformaldehyde for further research.

### 2.2.2. Micro-CT measurements

Each specimen was fixed in a cylindrical specimen holder. A high-resolution desktop cone beam scanner micro-CT (Bruker Skyscan, Germany) was used to scan slice thickness of 9 μm under 60 kVp and 417 μAn. According to the tooth extractive defect, the volume of 4 mm3 was selected as the region of interest (ROI). Next, 3D reconstruction analysis for this area was executed by NRecon software (Bruker, Germany). The parameters of bone mineral density (BMD) and mineralized bone volume fraction (BV/TV) were compared to assess the healing of alveolar socket bone in each specimen.

### 2.2.3. Histopathological analysis

After micro-CT analysis, the tissue blocks were immersed in 17% EDTA (Sangon Biotech Shanghai, China) solution (pH 7.4) for 3 weeks to decalcify. Then the decalcified tissues were embedded in paraaffin after gradient dehydration, 5 μm paraaffin sections were made and stained with H&E staining (Solarbio, Life Sciences, Beijing) and Masson’s trichrome (Solarbio, Life Sciences, Beijing). Then the images were photographed under a light microscope.

### 2.3. Statistical analysis

All experimental data were analyzed using GraphPad Prism version 8.0 statistical software, and the statistical differences among each group were evaluated using a t-test and one-way ANOVA test. Statistical significance was defined as “*” p < 0.05, “**” p < 0.01, “***” p < 0.001.

### 3. Results

#### 3.1. Surface characteristics and rhBMP9 adsorption potential on bioglass

The low-magnified SEM found that bioglass exhibited a 3D rough surface structure (Figure 1A), which would be more conducive to cell adhesion and particle absorption. The high-magnified SEM images of bioglass illustrated numerous micropores on the surface (Figure 1B), similar to that in cancellous bone. The pore size was 75–300 μm, which significantly increased the surface area of the material. The ELISA kit was used to calculate the total adsorption and release of rhBMP9 on bioglass. It was observed that the adsorption rate of rhBMP9 was close to 90% after pre-soaking and then released slowly. From 15 min after soaking to the 10th day, the release of rhBMP9 decreased gradually, and nearly 50% of initial rhBMP9 was released from bioglass at 10 d (Figure 2).

#### 3.2. Cell viability, adhesion, and proliferation behavior of rhBMP9 soaked bioglass

The BMSCs viability, adhesion, and proliferation on bioglass incorporated rhBMP9 were assessed. It was found that bioglass decreased cell viability at 24 h (p < 0.01), but the combination with rhBMP9 slightly increased the cell activity (p < 0.05) (Figure 3). Similarly, the cell adhesion rate at 8 h (p < 0.001) and cell proliferation at 1, 3, and 5 d (p < 0.01) were decreased in the bioglass group alone versus the control group but increased with the additional of rhBMP9 by the cck-8 kit detection (p < 0.01) (Figure 4A, B).

#### 3.3. Cell osteoblastic differentiation result on rhBMP9 soaked bioglass

The BMSCs osteoblastic differentiation on bioglass combined with rhBMP9 was studied by ALP activity assay, Alizarin red staining, and real-time PCR. Bioglass was found to have a positive effect in inducing ALP, and when associated with rhBMP9, the ALP activity was further enhanced markedly (p < 0.001) (Figure 5). Through alizarin red staining experiment, thickly stained massive mineralized nodules were observed in the bioglass + rhBMP9 group at 14 d, which were more denser and evident than that in the bioglass group, indicating that rhBMP9 played an active role in cell mineralization ability (Figure 6A, B). The real-time PCR

### Table 1. PCR primers for genes encoding Runx2, ALP, OCN and GAPDH.

| Gene  | Primer sequence          |
|-------|--------------------------|
| mRunx2 F | aggcatctgatgctaaacaac    |
| mRunx2 R | ggtctcgctgtctaaactt     |
| mALP F | ggagaaggcaacacacaccaaca |
| mALP R | ccaaaacagagcactcaactca  |
| mOCN F | ccagacacatgagagccactc   |
| mOCN R | ccagtaggctgtcaggaattg   |
| mGAPDH F | aggtcggtgaagcggattg     |
| mGAPDH R | Tgtagccattgagtgtaggtc   |

### References

29. Fujisawa and colleagues
30. Bioglass/2 μg rhBMP9 + BG (n = 5), (iv) Bioglass + BG/2 μg rhBMP9 (n = 5). Either BG (cut in 2 mm diameter circle) or 2 mg of Bioglass were implanted directly in the incisive fossa and soaked with 2 μL of rhBMP9 solution or sterile saline (control), in which the amount of rhBMP9 was taken based on a series of studies on rhBMP9 compounds and various carrier materials by the Japanese scholar Fujisawa and colleagues [29, 30, 42, 43, 44]. Then, the surrounding mucosal tissue was pulled up and sutured. Six weeks after the procedure, rats were sacrificed, and maxillary alveolar bone tissue on the extraction side was dissected carefully and fixed in 4% paraformaldehyde for further research.
results demonstrated that bioglass alone had a limited influence on the expression of osteogenic genes. Although the additional use of rhBMP9 within scaffold did not affect Runx2 mRNA expression levels (\(p > 0.05\)) (Figure 7A), an attractive improvement in ALP about six times (Figure 7B) and OCN about three times (Figure 7C) mRNA expression levels was observed at 14 d (\(p < 0.001\)).

### 3.4. Micro-CT evaluation of bone regeneration in alveolar sockets

Each defect area was scanned and analyzed by micro-CT. It was revealed that after extraction of right maxillary incisors, bone augment was found in alveolar sockets of all groups (Figure 8A). The selection of the center of alveolar bone defect was made for 3D reconstruction. The images indicated a more distinct new bone mass in all bioglass samples than control samples (Figure 8B). Furthermore, the blank group displayed a lower level of bone mineral density (BMD) (\(p < 0.01\)), while no visible difference was found between any other group that contained grafts (Figure 9A). The bone volume fraction (BV/TV) of bioglass/rhBMP9 + BG and bioglass + BG/rhBMP9 samples were higher compared to blank (\(p < 0.01\)) and bioglass + BG samples (\(p < 0.05\)). However, few differences were observed irrespective of whether rhBMP9 was soaked on bioglass or BG (Figure 9B).

### 3.5. Histological observation of bone regeneration in alveolar sockets

After six weeks of tooth extraction in rats, the specimens were fixed and histologically observed by HE (Figure 10A) and Masson staining (Figure 10B). It was found that the new bone in the blank group and bioglass group alone were limited, bone collagen and capillaries were sparse, and there was more fibrous connective tissue. However, the density of capillaries and bone collagen in the bioglass/rhBMP9 + BG group and bioglass + BG/rhBMP9 group increased, providing a good blood supply for new bone regeneration. It shows that the application of rhBMP9 has a positive effect on bone regeneration. Interestingly, more pronounced, new bone collagen were observed at the bottom of the defects in all rhBMP9 soaked on BG samples. It explained that rhBMP9 was more likely to cooperate with collagen membrane than bone graft materials in achieving a more effective site preservation effect.

### 4. Discussion

Site preservation technique refers to the implantation of specific bone substitutes in the alveolar fossa to control pathological or physiological resorption of the alveolar bone [45]. After tooth extraction, immediate operating site preservation surgery could avoid complex bone grafting in the later stage and provide suitable 3D morphology and soft and hard tissue conditions for subsequent implant restoration based on numerous clinical studies [46, 47]. Nevertheless, the simple application of bone graft materials to raise alveolar ridge was insufficient; therefore, some researchers tried to improve this process through tissue engineering. Among them, the incorporation of biomaterials and growth factors has been the most common strategy. Searching for reliable scaffold materials to deliver growth factors for accomplishing better bone repair therapies became a research hotspot. In recent years, bioactive glass has become
increasingly popular in bone augmentation due to its excellent biological
activity and efficient osteogenic capacity [48]. Bioglass is a kind of sili-
cate artificial bone substitute material composed of oxides such as CaO
and SiO₂. It carries out ion exchange with bone and soft tissue under
body circulation and stimulates osteoblast differentiation and bone
mineralization by promoting the expression of osteogenic protein gene
and type I collagen [14]. Many in vivo experiments have verified that
new bone is produced in relatively large amounts when used in fresh
tooth extraction wounds or different types of bone defects [16, 49, 50].
When incorporated with different growth factors, the osteogenic power is
significantly enhanced [51, 52]. The bone substitute selected in this
study was an innovative product developed based on primary 45S5
bioactive glass. Its rough surface and porous structure were more
convenient for osteoblast adhesion and new bone tissue growth.

On the other hand, it can be used under different biological charac-
teristics by soaking functional growth factors, antibiotics, and so on [50,
51]. It also has bacteriostatic properties and is compatible with X-ray
examinations, making it well-fitted for all kinds of oral tissue defects. In
the past, BMP2 was regarded as the standard of growth factors to stim-
ulate bone regeneration [53], while BMP9 presented a more distinct
influence in the bone induction process recently. Fujioka-Kobayashi [54]
discovered that even at concentrations of less than 20 times, the osteo-
genic activity of rhBMP9 was still attractive than that of other rhBMPs,
and rhBMP3 or Noggin could not inhibit this activity. Similarly,
Kobayashi [55] evidenced that even the lower doses of BMP9 had higher
levels of osteogenic differentiation than BMP2. Saulacic [56] used
different carriers soaked in rhBMP2 or rhBMP9 to implant into the
extraction fossa of dogs to compare the healing effect. The results showed
that the rhBMP9 group had a more remarkable bone formation.

Regarding the side effects of using high-dose BMP2, it was suggested
that low-dose BMP9 with high osteopromotive potential is more likely to
provide a promising clinical relevant option. Therefore, the authors
chose to soak rhBMP9 onto bioglass to testify its feasibility as an appli-
cation tool for osteogenesis for the first time. The micropore structure of
bioglass is capable of adsorbing protein, as exhibited by scanning elec-
tron microscope, and Elisa further confirmed its suitability as a carrier.
It was viewed that about 90% of the initial rhBMP9 was soaked onto bio-
glass first and gradually released into the surrounding environment with

Figure 4. Adhesion and proliferation detection of BMSCs seeded on bioglass in comparison to bioglass + rhBMP9. BMSCs adhesion on the control group, bioglass group, and bioglass + rhBMP9 group at 8 h. (A) BMSCs proliferation on the control group, bioglass group and bioglass + rhBMP9 group at 1, 3 and 5 d. (B) (** denotes significant difference p < 0.05 *** denotes p < 0.01 **** denotes significantly higher than all other groups p < 0.001).

Figure 5. Alkaline phosphatase (ALP) activity detection of BMSCs seeded on the control group, bioglass group, and bioglass + rhBMP9 group at 7 d.

Figure 6. Alizarin red staining detection of BMSCs seeded on bioglass group (A) and bioglass + rhBMP9 group (B) at 14 d.
passage of time until about 50% was retained within bioglass at 10 days. This favored its appropriate template structure of slowly and sustainably releasing rhBMP9 with time. However, as the new bone repair materials based on 4S55 bioactive glass are different in chemical composition, production method, shape, and size, the osteogenic properties are also different. In this research, the behavior of cells suggested that bioglass decreased the vitality, adhesion, and proliferation of BMSCs, but the addition of rhBMP9 increased the activity and adhesion rate of BMSCs. The analysis of the reason may be related to the different composition of bioglass and the active regulation of rhBMP9 on BMSCs. Besides, the expression of osteogenic related genes (RunX2, ALP, and OCN) were markedly highlighted in rhBMP9/bioglass group than in the bioglass group and blank group on the 3rd and 14th day of mixed culture. Moreover, on the 7th and 14th day after culture, versus that with blank scaffold, the scaffold soaked with rhBMP9 apparently upregulated the expression of ALP and the formation of extracellular mineralized matrix. All these results may be attributed to the good osteoinductivity of rhBMP9, and bioglass may supply a more favorable environment for rhBMP9-involved bone regeneration. However, to know whether the composite of bioglass and rhBMP9 still has an osteogenic effect in vivo requires further research.

GBR technology has become the most frequently used bone augmentation procedure in dental clinics due to its ease of operation, less trauma, and long-lasting effects by binding various graft materials with
rhBMP9 groups were higher when compared to blank and bioglass only a small amount of promote new bone formation. From a histopathological point of view, groups, indicating that rhBMP9 can cooperate with scaffold materials to may be related to the high mineral density of bioglass. However, in terms of bioglass/rhBMP9 significantly higher than the blank group, which may be caused by the fact that rhBMPs were more easily adhered to collagen membrane [57, 58]. Thus, except grafts, as another sustained-release carrier, collagen membrane offered a new breakthrough for transmitting growth factors. The composite membrane overtakes the role of isolation barrier and is beneficial to guide and accelerate bone recovery. By surveying the attachment, proliferation, and differentiation of ST2 cells with rhBMP9 or rhBMP2 loaded on collagen membrane in vitro, Fujioka-Kobayashi [43] proved the rationality of collagen membrane as a carrier. Animal experiments [40] further revealed that the osteogenic capacity of rhBMP9 delivered by collagen membrane was better than bone grafts. Saulacic [39] also studied the ability of rhBMP9 to induce new bone formation in two different carrier systems (BO and BG) by using the GBR model of a rabbit skull defect, and the same conclusion was drawn. The present study is the first to use a rat tooth extraction site preservation model to study the behavior of rhBMP9 on alveolar ridge retention using two different carriers. By soaking 2 μg rhBMP9 on either bioglass or BG directly, the new bone formation was distinguished via Micro-CT and histological analysis.

The results of 3D reconstruction analysis showed that the bone mineral density of bioglass/rhBMP9+BG, bioglass + BG/rhBMP9, and bioglass + BG groups were significantly higher than the blank group, which may be related to the high mineral density of bioglass. However, in terms of new bone volume fraction, bioglass/rhBMP9+BG and bioglass + BG/ rhBMP9 groups were higher when compared to blank and bioglass + BG groups, indicating that rhBMP9 can cooperate with scaffold materials to promote new bone formation. From a histopathological point of view, only a small amount of fibrous tissue capsules were scattered in alveolar fossae of group I, indicating that the effect of autogenous repair was poor without intervention. The observation results of group II implied that using scaffold materials for merely constructing tissue engineered bone free of growth factors could not accomplish the purpose of site preservation predictable. But the formation of capillaries and collagen tissue in group III and group IV were more significant, which was uniform as the conclusion drawn in the first part and further proved that rhBMP9 had a positive impact on osteoblastic induction in vivo. Although there are no characteristics of newly formed bone such as osteoid, osteocytes and some lining cells, it can be found that the neovascularization and connective tissue formation of the experimental groups were more significant than those of the control groups by comparing the staining results of all groups. And the NB formation either in HE or Masson staining of all the groups were not obvious may be due to the short feeding time of rats before killed. Previous studies have shown that the optimal osteogenesis time of rats was about 6–8 weeks, so the choice of killing rats at the sixth week in this study may be the main reason for the lack of obvious osteogenesis. The regeneration and reconstruction of blood vessels and collagen is the first step in the process of osteogenesis. Due to the short time, BMP9 did not play a full induction role, so only neovascularization and connective tissue representing the new bone could be seen in the experimental groups which means that they were still in the early stage of bone regeneration. But they can also explain the experimental conclusion, and there should be more studies that extended time or more data to further demonstrate the conclusion.

Interestingly, all specimens in bioglass + BG/rhBMP9 groups instead of bioglass/rhBMP9+BG samples demonstrated more bone collagen regeneration than the best specimen in the bioglass + BG group that may be likely caused by the fact that rhBMPs were more easily adhered to collagen membrane. So far, there have been many studies on the successful delivery of growth factors by using collagen membrane as a carrier, even displayed a more pronounced bone defect repair [59]. Besides, some scholars have specially observed the adsorption characteristics of BMP on collagen membrane or bone tissue particles. Surprisingly, it was discovered that more proteins were reserved on collagen-based biomaterials after 10 days of release, more proteins were reserved on collagen-based biomaterials [55]. Kobayashi [60] reported that the presence of atelocollagen could improve growth factor adsorption and induce osteoblast differentiation behavior of NBM if served as a bone graft scaffold. In general, the integration of collagen in carrier materials may be the main reason to affect the adsorption and release performance.
of growth factors. Previous studies have reported that BMP9 and BMP2 use the classical BMP/Smad pathway or non-classical signal pathway such as Wnt/β-catenin to promote osteogenesis [31]. Different scaffolds affect the expression of some proteins in cells, but the specific mechanism related to this process is still not clear. These experimental results show that rhBMP9 enhances the osteoinductive properties of bioglass, while bioglass may interfere with the protein expression pathway. They interact and cooperate, which exhibits a sound effect on bone regeneration and provides a theoretical basis for further studying the combination of growth factors and bone grafts in alveolar bone tissue repair. It is believed that with the rapid development of scientific research and gene sequencing, the unique regulation mechanism of rhBMP9 will be clarified deeply.

The rational use of rhBMP9 and bioglass in GBR may provide an effective clinical solution to bone deficiency caused by alveolar bone atrophy after tooth extraction. However, many problems are yet to be solved, such as controlling the release time, appropriate concentration, action mechanism of rhBMP9, and whether it can be used in clinics. Further preclinical studies are necessary to determine whether other bone graft materials with collagen are more suitable to combine with rhBMP9 for better site preservation.

5. Conclusion

The findings of this study revealed that both bioglass and BG effectively induced bone formation with rhBMP9 in GBR surgery for ridge preservation. Moreover, BG-soaked with rhBMP9 achieved superior wound closure than bioglass-soaked with rhBMP9, indicating that collagen membrane may have more potential advantages than bioglass when combined with growth factors.

Declarations

Author contribution statement

PeiKai Shi: Conceived and designed the experiments; Wrote the paper.

WanTong Zhou: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

JingBo Dong, ShuJun Li: Contributed reagents, materials, analysis tools or data.

PengJun Lv, Chenxi Liu: Performed the experiments.

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