Original Article

Evaluation of a biodegradable graft substitute in rabbit bone defect model

XiaoBo Yang, Yong Li, Qiang Huang, Jing Yang, Bing Shen, FuXing Pei

Abstract

Objective: To evaluate a new biodegradable copolymer calcium sulfate/poly amino acid (CS/PAA) as a graft substitute for the repair of the surgically created cancellous bone defects in rabbits and its biological properties *in vivo.*

Materials and Methods: Cancellous bone defects were created by drilling holes in the unilateral lateral aspect of the femoral condyle of New Zealand white rabbits. Three groups were assigned: Group A rabbits were grafted with 80% CS/PAA and group B rabbits were grafted with 95% CS/PAA as two treatment groups; group C was sham-operation control group. To study the osteogenic capability *in vivo,* specimens were harvested at 4, 8, 12, and 16 weeks after implantation and were evaluated by gross assessment, X-ray, histological examination, and histomorphometry. In order to identify the molecular mechanism of bone defect repair, the expression of bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) was detected using Western blot at 4 weeks.

Results: Group A and group B showed more vigorous and rapid repair leading to regeneration of cancellous bone than sham-operation control group on gross observation, radiology, and histomorphometry. There was no significant difference between groups A and B. Morphological observation and histological examination showed that the copolymers degraded in sync with the new bone formation process. The expression of BMP-2 and VEGF in implantation groups was higher than that in control group by western blot.

Conclusion: These findings demonstrated that the novel biodegradable copolymers can repair large areas of cancellous bone defects. With its controllable degradation rate, it suggests that CS/PAA may be a series of useful therapeutic substitute for bone defects.

Key words: Biodegradable, bone defects, graft substitute, polymer

Introduction

Bone defect repair has been a problem of common interest of the clinical, biological, materials science, tissue engineering, and other fields. Generally, defects are grafted with autogenous bone, allogeneic (species) bone, and synthetic materials to promote bone repair.[1] However, the sources of autogenous bone grafts and allografts are limited. Furthermore, immune rejection and other issues resulting from bone transplantation are still unsolved, which leads to a variety of complications.[2] Synthetic bone biomaterials could partly overcome the disadvantage of autologous and bone allograft and be used in clinic widely. Among the various types of biomaterials, synthetic biodegradable polymers are of most interest for clinical use as bone substitutes and scaffolds because of degradability, biological safety, and biocompatibility.[3,4] For example, Matsushita *et al*.[5] developed a new biodegradable graft substitute, which appeared to be effective in enhancing the repair of both cancellous and cortical bone defects with osteogenic potential, by using porous beta-tricalcium phosphate (b-TCP) granules, bone morphogenetic protein (BMP), and a synthetic block copolymer composed of poly-d, l-lactic acid with randomly inserted *p*-dioxanone and polyethylene glycol (PLA-DX-PEG). Thus far, aliphatic polyester, polyanhydride, polyurethane, and poly amino acids are the most extensively studied biodegradable materials in bone repair.[6-8] In addition to their adjustable mechanical properties and degradability, they also can be used in conjunction with bone growth factors, which further promote bone reconstruction.[9-11]

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Calcium sulfate based composite of poly amino acid (CS/PAA) is one of the most suitable biomaterials which could promote the bone defect repair and has the following features: Adjustable degradation rate, good osteoconduction, is a nontoxic catabolite, and is easy to prepare. An earlier study showed that poly amino acid was a prospective biodegradable biomaterial with excellent biocompatibility which could be used in clinical applications.[12] A lot of experimental and clinical studies have confirmed the application of calcium sulfate as a scaffold material in bone tissue engineering.[13-15] Poly amino acid in simulated body fluid (SBF) or phosphate buffer solution (PBS) can be hydrolyzed into small molecular weight amino acid, and CaSO4 in SBF or PBS can be hydrolyzed into calcium ions and sulfate ions, both of which are totally nontoxic to the human body. In the present study, we established a series of biodegradable copolymer-calcium sulfate/poly amino acid graft substitute and investigated its potential and mechanism for bone defect repair. We employed gross assessment, X-ray examination, histopathology of biopsy, histomorphometry, and Western blot to evaluate the role of this material in bone defect repair and identify the potentiality of application and feasibility in clinical practice. Our data strongly suggest that this novel composite is a promising and ideal biomaterial for bone repair and reconstruction.

Materials and Methods

Material composition

CS/PAA was provided by the Sichuan National Nano Technology Co. Ltd., Province Chengdu, Sichuan. Two specifications of materials included 80% CS/PAA and 95% CS/PAA according to the proportion of calcium sulfate. The poly amino acid was composed of 6 g alanine, 7 g benzene alanine, 1 g glycine, 108 g 6-aminocaproic acid, 6 g proline, and 2 g lysine. Raw materials [appropriate proportion of calcium sulfate and poly amino acid] were dissolved together in water and were dehydrated at 150°C–160°C temperature. Using inert gas for protection, the raw materials were processed with a pre-polymerization reaction at 220°C in a molten state for 3 hours and then polymerization reaction at 230°C for 3 hours. By *in situ* polymer composite technology, poly amino acid and calcium sulfate were used as the composite materials without any catalyst and other additives. The composite materials were ground into powder less than 120 mesh after cooling down by nitrogen, reconciled into a paste with water (1:1), and then processed into granules (d=2.5 mm, h=2.5 mm) [Figure 1a]. The materials were sterilized by radiation for reserve at last.

Degradation studies *in vitro*

PBS (pH=7.4) was used as the medium. Ninety-six samples (48 each in groups A and B) were processed into granules (d=10 mm, h=10 mm) for study of degradation *in vitro*. Each specimen was placed separately in a sealed vial and immersed in about 5 ml PBS solution. All the sealed vials were kept in a shaking incubator at 37°C and the PBS solution was changed every week. At 1, 2, 3, 4, 5, and 6 weeks, the specimens were removed, rinsed with deionized water, and dried to a constant weight in vacuum for weight loss ratio measurement. The weight loss ratio was measured according to the equation:

$$\text{Weight lost} = [(W0 - W1)/W0] \times 100\%,$$

where W0 and W1 are the weights of the specimen before and after the hydrolytic degradation, respectively.

Animals and implantation

The "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws (e.g. the current version of the German Law on the Protection of Animals) where applicable.

Seventy two New Zealand white male rabbits (3 months of age, 2.0–2.5 kg body weight, from Experimental Animal Center of Sichuan University) were included in the study. Premedication with intraperitoneal injection of 10% chloral hydrate anesthesia at a dose of 2.5 mL/kg was given. After the skin preparation, a cancellous bone defect area (diameter=0.5 cm, depth=1.0 cm) was drilled using a 5-mm-diameter trephine across the lateral aspect of the femoral condyle. The defect was placed within the epiphysis, avoiding communication with the knee joint cavity [Figures 1b and c]. Intramuscular injection of antibiotics (Cefazolin, 100 mg/kg) was given immediately after surgery and on the first day post operation.

Bone defect was grafted with the following materials: 80% CS/PAA (group A, n=24) and 95% CS/PAA (group B, n=24). Control group (group C, n=24) was under the same operation, but was not implanted with any material. Animals were euthanized at 4, 8, 12, and 16 weeks. Six animals were allocated to each group at each time point for gross observation, X-ray analysis, histological examination, histomorphometry, and Western blot testing.

Gross assessment

The animals were euthanized at each time point, and the specimens were collected by cutting down from about 5 cm above the femoral condyle and were cleaned of connective tissue and fat for observation. We collected information including inflammation, material degradation, and bone defect repair for gross evaluation. Then, the specimens were prepared for X-ray. The evaluation was made by two professional clinical orthopedists who were blinded to the study.

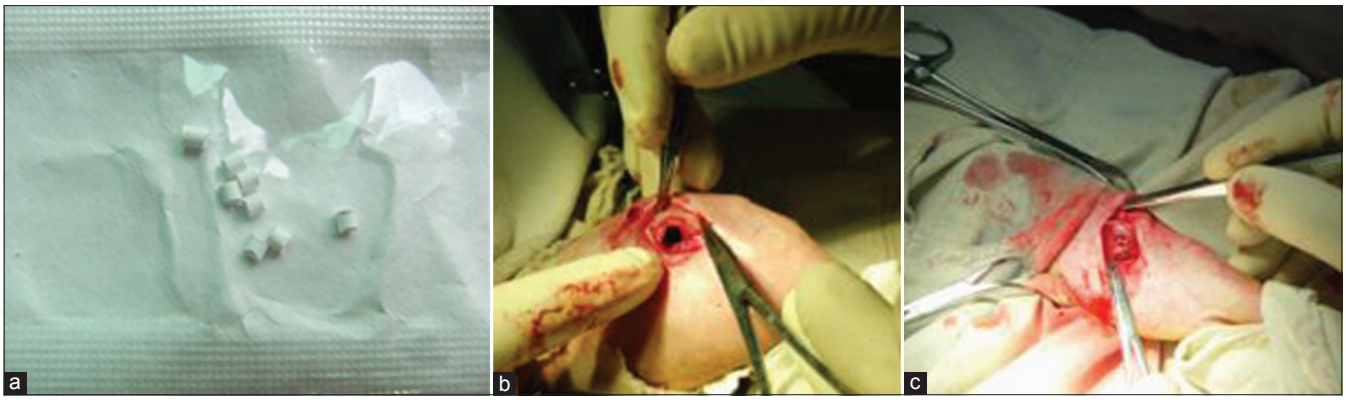

Figure 1: The materials and rabbit model with bone defect: (a) materials; (b) model of rabbit femoral condyle bone defect; (c) defects were grafted by CS/PAA

X-ray analysis

At the end of 4, 8, 12, and 16 weeks, the animals were euthanized and the specimens were analyzed by conventional radiography at the Radiology Section of West China Second Hospital of Sichuan University, and completed by AGFA computed radiography system (Computer Radiography, ADC-SOLO; ADC25.0). The exposure conditions were: 50 kV, 55 mA, exposure time 0.3 sec. X-ray images were assessed by two trained radiologists who were blinded to the study.

Histological and histomorphometric assessment

The specimens were collected immediately after X-ray and fixed in 10% formaldehyde for histological and histomorphometric assessment. Then, they were embedded in methylmethacrylate after dehydration in graded series of ethanol. Nondecalcified, 5-μm-thick longitudinal sections were made using microtome (Leica, SM 2500E). All the samples were stained with hematoxylin and eosin for observing the status of the implants and the cellular response of the hot bone, or stained with improved special Masson trichrome for histomorphometric assessment. The histologic sections were photographed using a Nikon Microphot microscope (Nikon, eclipse e600) attached with a digital color camera (Nikon, dxm 1200). Then, the digital images were processed using a semi-automatic digital image analysis system (OsteoMeasure; OsteoMetrics, Inc., Decatur, USA). All the measurements were performed in the five different transverse sections of each sample. The newly grown bone quality was measured according to the principle and methodology approved by American Society of Bone and Mineral Research (ASBMR).[16] The static parameters measured included bone volume (BV/TV, %) in order to quantify the amount of newly formed bone.

Lysate preparation and Western blotting

At 4 weeks after surgery, the implant region was dissected immediately after X-ray test, and the part of each tissue sample was isolated, weighed, and ground into powder in liquid nitrogen. The tissue powder was homogenized in pre-cooled radioimmunoprecipitation assay (RIPA) (200 μl per 100 mg tissue) and the total protein was extracted by protein extraction kit (Biovision, CA, USA). The protein concentration was determined using Micro BCA protein assay kit (PIERCE 23227). Equal amounts of proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Poll). The membranes were incubated with primary antibodies against BMP2 (Abcam ab6285) and vascular endothelial growth factor (VEGF; Santa sc-365578). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:20,000; PIERCE, Rockford, USA) and an ECL detection system (PIERCE, USA). Signals were quantified using NIH ImageJ 1.63 Software.

Statistical analysis

All experimental results were expressed as means ± standard deviation. Differences were considered significant for $P<0.05$. Differences in histomorphometric related trait value among groups were assessed by chi-square test at each time point. One-way analysis of variance (ANOVA) was used to determine the differences in BMP-2 and VEGF expression between groups at 4 weeks.

Results

In vitro degradation rate

The results showed that materials degraded dramatically in the first 2–3 weeks; 80% CS/PAA degraded 50.93% at the end of 3 weeks while 95% CS/PAA degraded 58.86%. The degradation rate slowed down and became smooth. At the end of 6 weeks, 80% CS/PAA degraded 63.27% while 95% CS/PAA degraded 74.66%. As we reduced the proportion of calcium sulfate in the copolymer, the degradation rate decreased [Figure 2].

Gross assessment

None of the animals died during the study. The specimens did

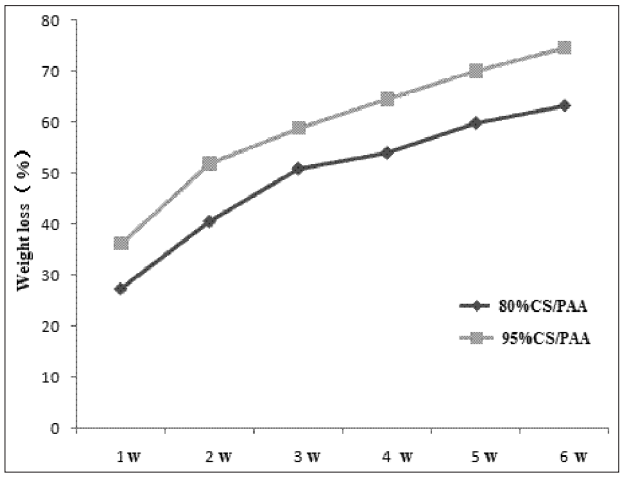

Figure 2: The graph showing comparision of degradation rate of 80% CS/PAA and 95% CS/PAA *in vitro*

not show any evidence of tissue infection and inflammation on gross observation [Figures 3a and b]. The materials got incorporated intimately with the surrounding host bone. The boundary between the materials and adjacent host bone became indistinct with time. In the control group, the specimens did not totally heal at the end of the experiment. At the early stage of bone repair, the area of defect was filled by blood clot (4 weeks after operation) and fibrous soft tissue (8 weeks after operation). At later stage of bone repair, partial new bone was observed and most of the defect was still filled by fibrous soft tissue [Figure 3c]. In the implantation groups, defects and materials were no longer visible on macroscopic examination at 16 weeks after the operation. Biomaterials were partly degraded at 4 weeks after operation. Half of 6 in group B (95% CS/PAA) and 1of 6 in group A (80% CS/PAA) were totally degraded at 12 weeks after operation. Specimens in the implantation groups revealed a substantial fill of repair tissue in bone defect regions with only a slight depression at the defect site visible at 12 weeks after the operation, while one specimen (1/6) in both the implantation groups got absolutely repaired. All the specimens in both the implantation groups showed complete repair of bone defect at 16 weeks after the operation [Figure 3d–f].

X-ray analysis

After 4 weeks of operation, X-ray analysis demonstrated high levels of bone formation in defects grafted with 80% CS/PAA and 95% CS/PAA, obvious high-density spots of regions were observed, and the defect margins became irregular. In the control group, bone defect did not show any appreciable bone formation and the defect margins were smooth. At 8 weeks after the operation, in groups A and B, the representative radiographs revealed signs of bone repairing, while bone defect was clearly visible in the control group. At 12 weeks after surgery, most of the regions with bone defects could not be observed in groups A and B and one specimen of both the treatment groups

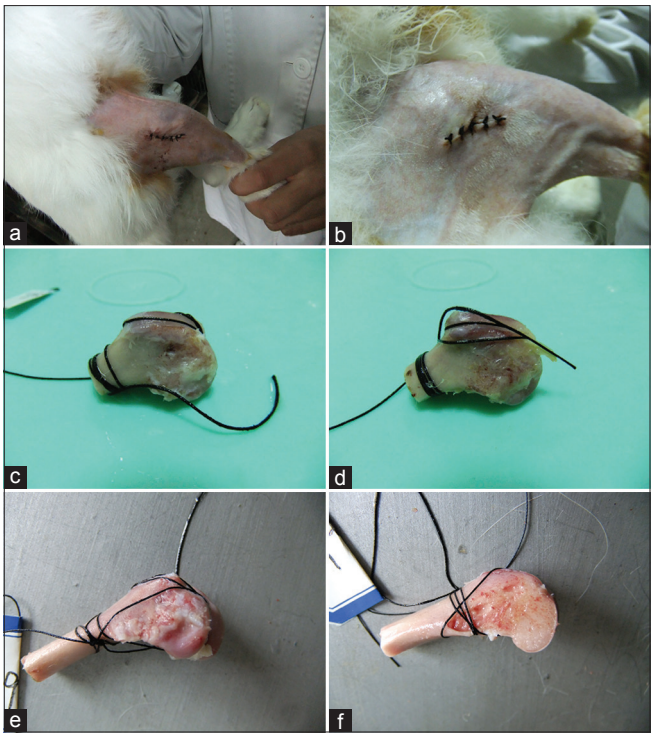

Figure 3: Gross observation after surgery: (a and b) group A (80% CS/PAA) and group B (95% CS/PAA), 7 days after implantation; (c) specimen of group C (control group) at 16 weeks; (d) specimen of group B (95% CS/PAA) at 16 weeks; (e and f) specimen of group A (80% CS/PAA) at 16 weeks

even showed completely repaired bone defect, while the defect was still visible in the control group. At 16 weeks after surgery, the boundary between the newly formed and normal bone almost disappeared and the bone defects were totally repaired in groups A and B. Whereas in the control group there was only a small amount of new bone formed in the defects and the cavity in bone defect region was still visible in all specimens at 16 weeks [Figure 4]. There was no significant difference between groups A and B in bone repair at each time point after the operation.

Histological and histomorphometric findings

Minimal inflammation was observed in all specimens as was also observed in gross morphology. Histological evidence further supported the X-ray findings [Figure 5]. At 4 weeks after the operation, the materials had started to degrade, newly formed collagen tissue was found around the fragment materials and juvenile osseous ingrowth was visible in both groups A and B, and osteocytes were obviously observed within the bone matrix. Control group showed sparse osteogenesis and the defective region was filled with blood clot. At 8 weeks after the operation, the bone ingrowth was accelerated; primarily developing woven bone, fibrous connective tissue, and the rest of the materials were observed in the implanted groups; osteoblast differentiation and juvenile vascularization were also visible; and newly formed trabecula bone became more compact. At 12 weeks after the operation, the implanted

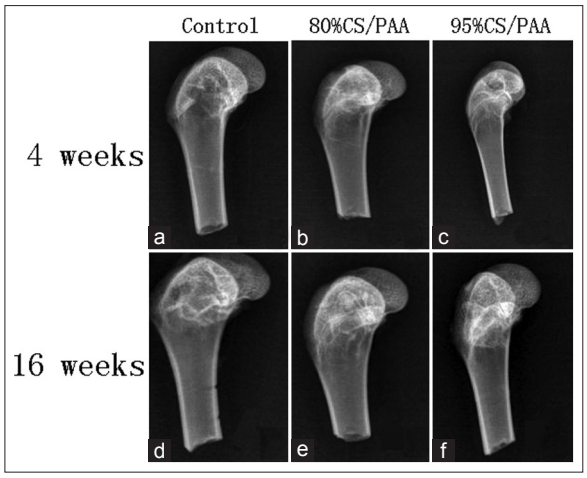

Figure 4: X-ray images according to the three groups and time points (a, b, c at 4 weeks and d, e, f at 16 weeks) are presented here. Demonstrate the differences in the cacellous bone defect repair of each group, with group A(80% CS/PAA) and group B(95% CS/PAA) show more vigorous and rapid repair than group C(control group)

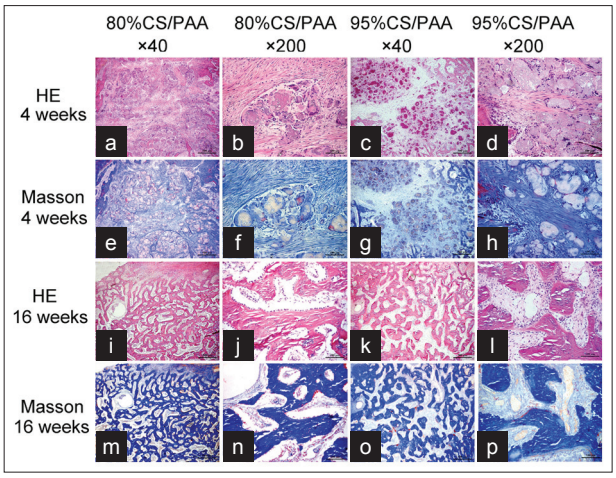

Figure 5: Histological micrographs of specimens from each group at 4 and 16 weeks after surgery: (a and b) 80% CS/PAA, H and E, ×40 and H and E, ×200, 4 weeks; (c and d) 95% CS/PAA, H and E, ×40 and H and E, ×200, 4 weeks; (e and f) 80% CS/PAA, Masson ×40 and Masson ×200, 4 weeks; (g and h) 95% CS/PAA, Masson ×40 and Masson ×200, 4 weeks; (i and j) 80% CS/PAA, H and E, ×40 and H and E, ×200, 16 weeks; (k and l) 95% CS/PAA, H and E, ×40 and H and E, ×200, 16 weeks; (m and n) 80% CS/PAA, Masson ×40 and Masson ×200, 16 weeks; (o and p) 95% CS/PAA, Masson ×40 and Masson ×200, 16 weeks

groups showed a notable amount of guided bone formation with osteoblasts presented in the trabecula compared to the control group. The materials induced increased osteogenesis with the newly formed bone filling the cancellous bone defective region. Three of six materials in group B degraded completely while degradation in group A was delayed by approximately 2–3 weeks. At 16 weeks after the operation, representative images demonstrated the regenerated bone with typical structure of matured bone in the defective region in both implantation groups, and all materials were absolutely degraded. In the control group, the defect was partially filled with new trabecula bone and most of the region of defect was filled by fibrous tissue.

Bone volume (BV/TV, %) is an indicator of the amount and quantity of the new bone formation. Figure 6 shows the histomorphometric measurements of defect region of each group from 4 to 16 weeks. BV/TV values increased from 4 to 16 weeks in all the groups. The value of BV/TV of groups A and B was significantly higher than in the control group at each time point after the operation ($P<0.05$). As for groups A and B, there was no significant difference in 8, 12, and 16 weeks, but a significant difference was found 4 weeks after the operation.

Expression of BMP-2 and VEGF

During the natural process of bone defect healing, BMP-2 and VEGF are generally expressed at a high level in the early stage. In the present study, the expression of BMP-2 and VEGF in both treatment groups was upregulated compared with control group ($P<0.01$) at 4 weeks [Figure 7]. Between groups A and B, there was no significant difference in the expression of BMP-2 and VEGF ($P>0.05$).

Discussion

Bone defect caused by trauma, infection, tumor resection, and congenital diseases has been a common clinical orthopedic problem. Current treatments include bone grafting by autologous bone graft, allograft, or xenograft bone transplantation, and using other biomedical materials.[17] Although autogenous bone graft treatment is satisfactory, its clinical use is still limited due to the donor and recipient site complications.[18] Allogeneic or xenogeneic bone grafts show rejection and may cause spread of viral diseases such as hepatitis and HIV. Tissue engineering and other techniques such as masquelet have gained a lot of interest among the scientists and surgeons, but until now, they have not been widely used in clinical applications.[19] Therefore, development of artificial biomaterials suitable for bone defect repair is an important subject in the field of medicine and materials science.[20-22] Calcium sulfate is widely used as a graft material in clinical subjects. It has been proved previously that calcium sulfate has good compatibility with human tissue and an excellent biodegradability.[23,24] However, its clinical applications are limited by its disadvantage of rapid degradation and brittleness.[25] For overcoming the drawbacks of mineral bone graft substitutes, several composite materials with natural and synthetic biodegradable polymers have been tested. D'Ayala *et al*. synthesized a new calcium sulfate based composite containing alginate and *N*-succinyl-chitosan as polymeric components and demonstrated it to be suitable for biomedical applications and easy to use for the clinicians.[26]

Calcium sulfate/poly(amino acid) is a series of copolymers which have excellent properties such as controllable degradation rate, good osteoconduction, and good biocompatibility. It is well known that if the graft substitute degraded over quickly or too slowly, it becomes unfit for bone defect repair. The best degradation pattern is that of a graft substitute that degrades in accordance with the new bone formation.[27] As for the special position and size of bone defects, there was a specified demand on the property and degradation rate of material. In our previous study, we found that 80% CA/PAA and 95% CA/PAA were suitable for defect repair of cancellous bone. In the present study, the degradation experiment *in vitro* showed that the biomaterial degraded rapidly in the first couple of weeks mainly because degradation mainly involved the dissolved inorganic surface of the material at this stage; then the degradation rate decreased and became smooth because later the degradation mainly constituted the graded hydrolysis of the long-chain molecules, which would take much more time. The whole degradation process of the material was corresponding with the degradation pattern that we hypothesized. Therefore, we can control the degradation rate by regulating the proportion of calcium sulfate in the material. Poly amino acid showed a strong biological activity compared with other biomaterials; its catabolites are amino acids, H_2O, or other small molecules which are safe for the body. As a graft substitute, the amino group can increase the mutual affinity that helps the cells adhere to the materials tightly.[28,29] The composite biomaterial of calcium sulfate/amino acid polymer, incorporating the merits of both, is supposed to provide a source of quality inorganic calcium and amino acids for tissue repair. Therefore, with good bone conductivity, biodegradability, and good biocompatibility,

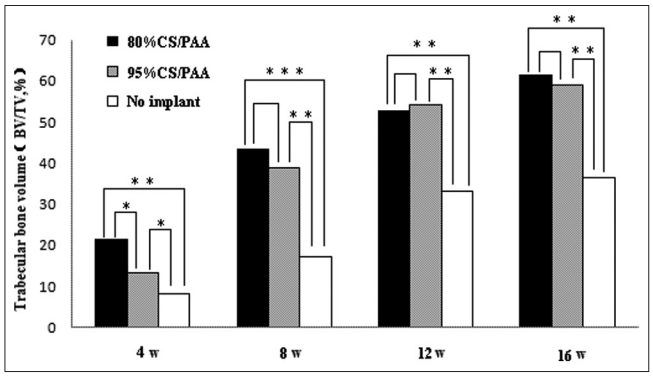

Figure 6: A bar diagram showing newly formed bone volume to total volume ratio (BV/TV, %) of each group, evaluating the quantity of new bone formation. The bone volume (BV/TV, %) of both the implantation groups is greater than that of group C (control group) at 4, 8, 12, and 16 weeks after surgery (**P*<0.05, ***P*<0.01, ****P*<0.001). The BV/TV showed no significant difference between group A (80% CS/PAA) and group B (95% CS/PAA) (*P*>0.05) at each time point except at 4 weeks(**P*<0.05)

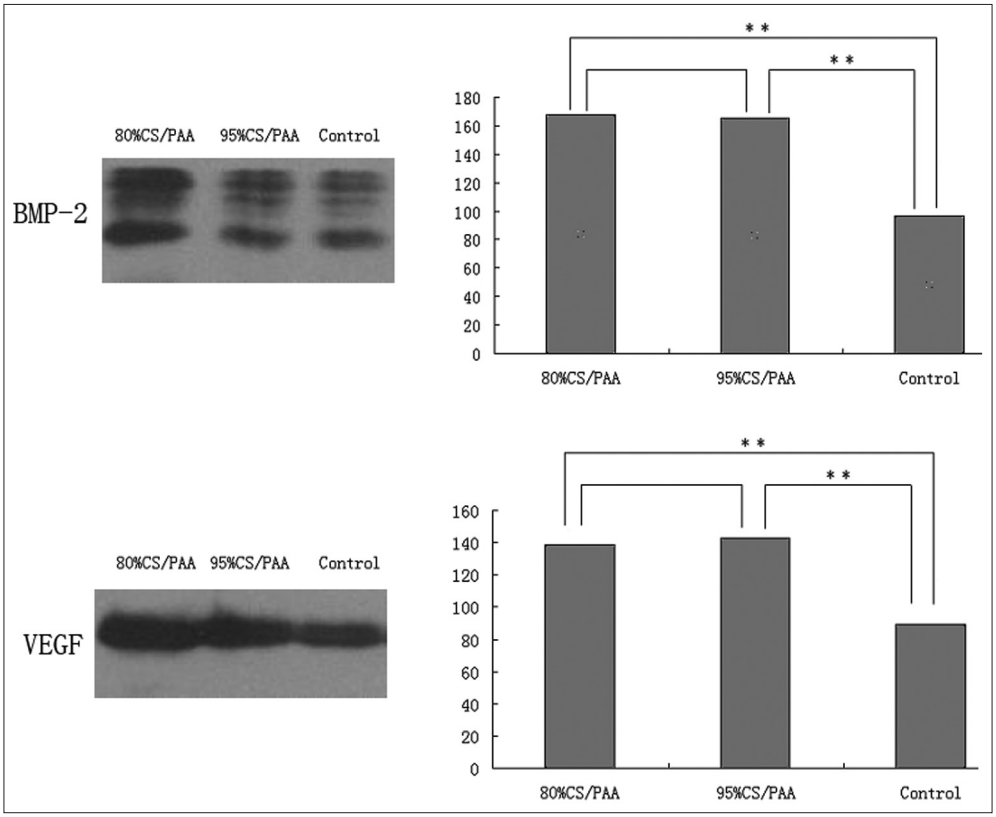

Figure 7: Western blot analyses of the expression of the osteogenic growth factor bone morphogenetic protein-2 (BMP-2) and angiogenic growth factor vascular endothelial growth factor (VEGF). There was a significant difference between the two implantation groups and the control group (***P*<0.01)

it has great potential value in the clinical orthopedic use.

In the present study, we further demonstrated that these porous biomaterials show a good performance in bone formation and have excellent biocompatibility *in vivo*. Based on our data and the information from previous studies, there are three properties of CA/PAA indicating that it is suitable for bone defect repair. The first property is absorption. In the previous studies, many synthetic biomaterials showed good osteoconduction and biocompatibility for bone defect repair. However, the degradation rates of most of the materials were uncontrollable.[30-32] In this study, CA/PAA has been found to have an advantage of controllable degradation rate by regulation of the proportion of the CS. Test of degradation rate *in vivo* showed that both the materials could be degraded dramatically in 6 weeks, which corresponded with the procedure of new bone formation. Degradation of biological material provides a space for new bone growth, and also catabolites of amino acids and calcium can supply the bone matrix for new bone formation. All these contribute to bone repair. The second property is biocompatibility. Gross observation and histological evidence demonstrated that CS/PAA and its catabolites are totally nontoxic, and have good biocompatibility and affinity with bone tissues and cells. The third one is osteoconduction and osteoinduction. Vascular and osteoblasts can ingrow into materials from surrounding tissue because porous materials provide a rough interface and adequate space. X-ray and histological results showed that bone defects were totally repaired at 16 weeks in the implantation groups while the cavity of bone defect region was still visible in sham-operated control group; this indicated that CS/PAA enhanced bone healing in cancellous defect.

Furthermore, our results showed that the expression of BMP-2 and VEGF was upregulated in the CS/PAA groups compared to that in the control group. It is well known that BMPs, including BMP-2, BMP-4, and BMP-7, have been used to induce bone formation and to repair bone defects. BMP-2 is mainly used to induce differentiation of osteogenic mesenchymal cells into osteoblasts and chondrocytes and produce new bone.[33] VEGF, the best-characterized angiogenic factor, plays an important role in bone growth and fracture healing via the endochondral ossification pathway.[34] VEGF can participate in the metabolism of bone formation through paracrine pathway.[35] In addition, VEGF can also act on osteoblasts to express flt-1 receptor which can increase the mobility and differentiation function of osteoblast. This suggests that CS/PAA promoting bone repair is involved in BMP and VEGF signal pathway.

In conclusion, CS/PAA is a potential therapeutic substitute for bone defects. Our study indicates that CS/PAA has a specific property of controllable degradation rate *in vitro* and promotes the healing of critical size bone defects *in vivo*. With features of controllable degradation rate, good osteoconduction, and histocompatibility, CS/PAA is suitable as a resorbable material able to induce bone repair in critical size defects.

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