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Biocompatibility of differently proportioned HA/PLGA/BMP-2 composite biomaterials in rabbits

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ABSTRACT. The aim of this study was to assess the histocompatibility of hydroxyapatite (HA)/poly(lactic-co-glycolic acid) (PLGA)/bone morphogenetic protein-2 (BMP-2) composite materials in rabbits. Thirty healthy New Zealand white rabbits were randomly divided into 3 groups (N = 10). HA/PLGA/BMP-2 composite materials with the HA/PLGA proportions of 1:1, 1:2, and 1:3 were implanted in the animals, which were subsequently sacrificed on the 30th and 60th days post-implantation to allow for differences in routine blood and biochemical indices to be assessed between the animal groups. The degree of biomaterial degradation was also assessed in the three groups. Thirty and 60 days after the implantation of titanium plates and composite materials, no rabbits succumbed to inflammatory reactions, adverse reactions, abnormal blood routine and biochemical indices, or unstable liver functions. The presence of newborn tissues was identified within the 60 days post-implantation. No significant differences were observed between the three groups ($P < 0.05$). The wide clinical application of HA/PLGA/BMP-2 composite biomaterial, which is highly compatible with rabbits with no apparent effects on the animals, is highly feasible.

Key words: HA/PLGA/BMP-2 composite material; Histocompatibility; Bone tissue engineering

INTRODUCTION

After implantation, an eligible biomaterial should be compatible with the host tissues and should maintain normal physiological activities by not obviously injuring organisms or causing fluctuations in routine blood and biochemical indices (Sagar et al., 2012). Compatibility testing is generally required before clinical application, since biomaterials, unlike other high-tech materials, ought to be compatible with the blood and tissues of target hosts. Currently, the aim is to develop materials that only induce mild inflammatory reactions without bringing about thrombosis or biotoxicity (Xia et al., 2012; Nguyen et al., 2013); however, individual biomaterials cannot cater to all needs, and thus composite materials with versatile properties are required. In this study, New Zealand white rabbits were implanted with composite biomaterials made up of different proportions of hydroxyapatite (HA)/poly(lactic-co-glycolic acid) (PLGA)/bone morphogenetic protein-2 (BMP-2). The aim of the implantations was to assess the blood and biochemical indices after clinical reactions, and to determine the proportions that give rise to the optimum histocompatibility.

MATERIAL AND METHODS

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the General Hospital of Shenyang Military Area Command of Chinese PLA, Rescue Center of Severe Wound and Trauma of Chinese PLA. All surgery was performed under anesthesia, and all efforts were made to minimize animal suffering. All materials were purchased from Sigma (St. Louis, MO, USA). Pathological sections were prepared by a Thermo machine (Waltham, USA). Microscope was bought from Olympus (Tokyo, Japan).

Experimental animals

Thirty healthy New Zealand white rabbits were randomly divided into 3 groups (N = 10). The basic information of the animals in each group is summarized in Table 1. The genders and body weights of the animals did not differ significantly ($P > 0.05$) between the three groups.

Table 1. Details of experimental animals in each group.

	N	Female	Male	Average body weight (kg)
Group A	10	4	6	2.6
Group B	10	3	7	2.5
Group C	10	5	5	2.5
Total	30	12	18	2.53

Experimental materials

HA/PLGA/BMP-2 composite biomaterials (15 x 4 mm), proportions of HA/PLGA: 1:1, 1:2, 1:3; titanium plate (external fixation); drugs: pentobarbital, iodine tincture, medical-grade ethanol, and penicillin; apparatus: syringe, surgical scissors, suture, devices for preparing sections, and microscope.

Experimental method

A schematic detailing the experimental procedures used in this study is shown in Figure 1.

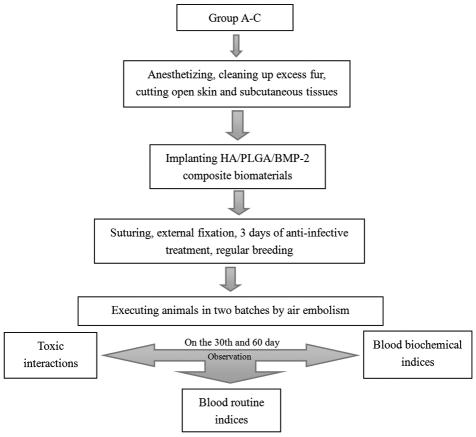

Figure 1. Schematic describing the experimental method used in this study.

Tailoring of biomaterials

HA/PLGA/BMP-2 composite biomaterials with different proportions of the individual components were tailored into blocks (15 x 4 mm), immersed in medical-grade ethanol for 30 min, washed three times with PBS (1 min each), and sterilized under UV light.

Implantation of biomaterials

After being anesthetized with pentobarbital by intravenous injection (15 mg/kg body weight), the fur on the femurs of the animals was cleared and the skin and subcutaneous tissue were cut open. The prepared biomaterials were then implanted into the exposed area and medical-grade ethanol or iodine tincture were used for sterilization when necessary (Figure 2) (Testori et al., 2012).

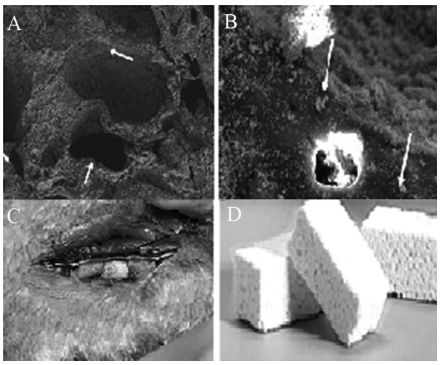

Figure 2. Implantation of biomaterials. **A.** External morphology of the PLGA/HA composite, which is highly porous with pore sizes of 30-1000 μm. **B.** SEM analysis of the PLGA/BMP-2/HA composite with arrows indicating pores of various sizes. **C.** Uniform distribution of ceramic particles on the polymeric matrix. **D.** HA particles.

Post-surgical treatment

After wound closure by suturing, the rabbit femurs were externally fixed with titanium plates. The animals then underwent anti-infective therapy (intravenous injection of penicillin) for 3 days after surgery. The physiological activities of the rabbits were observed after regular breeding, and animals were sacrificed by air embolism in two batches: 30 and 60 days post-surgery. Tissues were sampled and processed into sections to allow for potential pathophysiological changes to be assessed.

Routine blood indices

Routine blood indices including red blood cell count, white blood cell count, platelet count, and hemoglobin levels were measured. Hemocytes were counted by dropping diluted blood onto plates.

Biochemical blood indices

The levels of ions, glucose, proteins, enzymes, hormones or metabolites including glutamic-pyruvic transaminase, urea, total bilirubin, cholesterol, triglyceride, creatine kinase, and alkaline phosphatase were measured in the blood (Jose et al., 2009; Niu et al., 2009).

Toxic interactions

The compatibilities of the biomaterials with blood and tissues after implantation were assessed by staining and analyzing sections of host organs such as liver and kidney.

Visual assessment

Surface roughness was quantitatively analyzed using a “New View 5000” white-light scanning interference microscope.

Statistical analysis

All data were analyzed by SPSS 17.0 and differences between groups were analyzed by the *t*-test. Differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

Routine blood examination

All the rabbits survived and exhibited normal physiological activities and blood indices. Within the first 30 days, the animals in all three groups experienced mild inflammatory reactions and increased neutrophil levels. From the 30th to the 60th days, inflammatory cell numbers decreased. In Group A, for example, neutrophil levels dropped from $5.24 \pm 1.02 \times 10^9$/L to normal levels. Overall, the results across the three animal groups did not differ significantly at day 60 post-implantation ($P > 0.05$) (Table 2).

Table 2. Routine blood examination results from day 60 post-implantation.

Group	N	RBC (x10⁹/L)	WBC (x10⁹/L)	NE (x10⁹/L)	NE percentage (%)
Group A	5	5.02 ± 0.83	7.79 ± 0.21	5.24 ± 1.02	67.2
Group B	5	5.11 ± 0.28	7.80 ± 0.09	5.04 ± 0.52	64.6
Group C	5	5.08 ± 0.33	7.77 ± 0.11	5.17 ± 0.78	65.9
t		2.625	1.721	2.271	1.582
P		0.9728[1]	0.9836[2]	0.9442[3]	0.9628[4]

[1, 2, 3, 4]P > 0.05: no significant differences. RBC = red blood cell; WBC = white blood cell; NE = neutrophil.

Blood biochemical examination

The animals in the three groups were found to exhibit normal blood biochemical indices at each time point, with no significant inter-group differences (P > 0.05). On day 60 post-implantation, for example, the TB levels in Groups A, B, and C were found to be 13.02 ± 2.63, 12.85 ± 2.92, and 13.12 ± 2.37 μM, respectively (no significant differences: t = 1.781, P = 0.995; Table 3).

Table 3. Blood biochemical indices on day 60 post-implantation.

Group	N	CHO (mM)	GLU (mM)	TG (mM)	ALP (U/L)	CK (U/L)
Group A	4	2.02 ± 0.32	6.92 ± 0.27	1.46 ± 0.22	247.94 ± 77.25	403.81 ± 63.24
Group B	4	1.98 ± 0.33	6.72 ± 0.37	1.42 ± 0.26	238.29 ± 79.59	407.76 ± 66.12
Group C	4	1.94 ± 0.27	6.82 ± 0.29	1.44 ± 0.30	248.29 ± 75.83	405.66 ± 69.33
t		1.296	1.682	1.271	-3.291	-4.902
P		0.825[1]	0.999[2]	1.000[3]	0.729[4]	0.864[5]

[1, 2, 3, 4, 5]P > 0.05: no significant differences. CHO = cholesterol; GLU = glucose; TG = triglyceride; ALP = alkaline phosphatase; CK = creatine kinase.

Observation of pathological sections

On day 60 post-implantation, the animals in all three groups exhibited stable liver function and no liver damage (Figure 3). Moreover, no inflammatory cells were identified at the implantation sites (Figure 4). The presence of newborn tissues suggests that tissue repair was initiated following implantation.

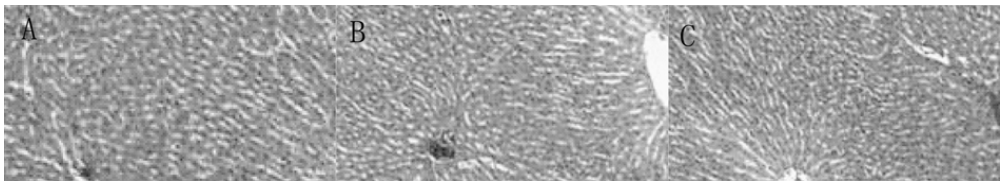

Figure 3. Liver tissue sections from each animal group. **A.-C.** Groups A-C, respectively.

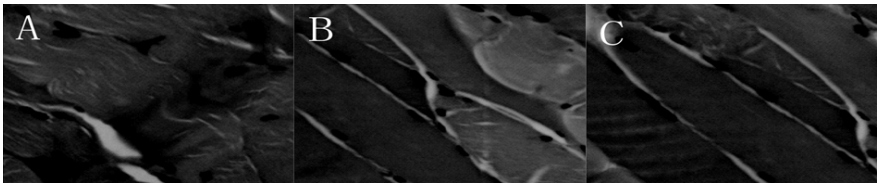

Figure 4. Soft tissue sections from each animal group. **A.-C.** Groups A-C, respectively.

Visual assessment of bone sections by SEM

Bone sections of the three groups are shown in Figure 5. Figure 5A shows an area under the crack in the cell layer within the pore wall of the PLGA/BMP-2/HA composite seeded with cells, which represents the formation of a layer of tissue within the area under a crack. The 1:1 composite (Figure 5B) is highly porous with the pore sizes varying from 40 to 1000 μm. The inner surface of the composite is also highly porous with pore sizes varying from 10 to 90 μm, as indicated by white arrows. The pore walls are significantly ($P < 0.05$) thinner than those of the 1:1 composites and are indicated by the black arrows. The average pore wall thickness of the 1:1 composite is 85 μm. The 1:2 composite (Figure 5C) is highly porous with pore sizes varying from 60 to 1000 μm. The inner surface of the composite is also highly porous with pore sizes varying from 10 to 40 μm, as indicated by the white arrows. The pore walls are significantly ($P < 0.05$) thicker compared to those of the 1:1 composites and are indicated by black arrows. The 1:3 composite (Figure 5D) is highly porous with pore sizes varying from 30 to 1000 μm. There are no inner pores, as indicated by the white arrow. The pore walls are significantly ($P < 0.05$) thicker than those of the 1:1 and 1:2 composites and are indicated by black arrows. The average pore wall thickness of the 1:3 composite was found to be 182 μm.

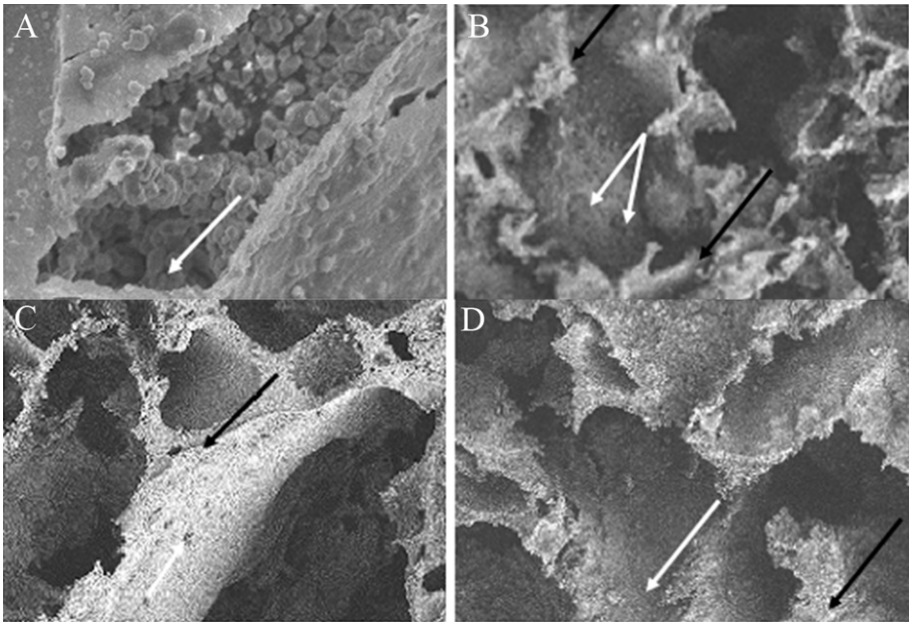

Figure 5. SEM analysis of bone sections. **A.** Area under a crack in the cell layer within the pore wall of PLGA/BMP-2/HA composite seeded with cells, describing the formation of a layer of tissue within the area under a crack. **B.** External morphology of 1:1 composite. **C.** External morphology of 1:2 composite. **D.** External morphology of 1:3 composite.

Observations from white-light scanning interference microscopy

White-light scanning interference microscopic observation results of the three groups are exhibited in Figure 6, with arrows indicating the scanning positions.

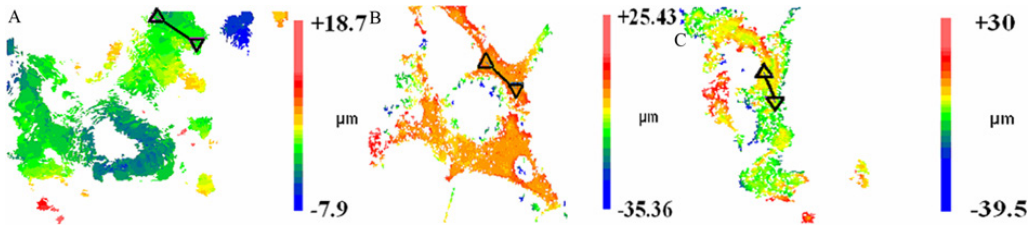

Figure 6. Scanning images from the animals of the three groups. The positive values correspond to the peaks and the negative values correspond to the valleys. The peaks are indicated by the green, yellow, and red areas. The valleys are indicated by the blue areas. **A.** Group A, the color contour is represented by the micron scale and varies from -7.9 to +18.7 μm. **B.** Group B, the color contour is represented by the micron scale and varies from -35.36 to +25.43 μm. **C.** Group C, the color contour is represented by the micron scale and varies from -39.5 to +30 μm.

DISCUSSION

After 60 days of post-surgical rehabilitation, none of the rabbits were observed to be suffering from evident clinical symptoms. The degradation of the implanted HA/PLGA/BMP-2 composite biomaterials induced inflammation by triggering changes (e.g., pH) at the implantation sites, which were reversed after thorough metabolism. In the meantime, the concomitant fibroblast proliferation and newborn tissues promoted bone repair and reconstruction. These materials are therefore applicable to bone tissue engineering owing to their high biocompatibility (Bhattarai et al., 2008; Kharaziha and Fathi, 2010; Lin et al., 2012), a characteristic feature of analogous materials. Of the three materials assessed in this study (Ji et al., 2010; Lee et al., 2011), HA [$Ca_{10}(PO_4)_6(OH)_2$] is widely distributed in human tooth enamel (present at over 96%) and is highly biocompatible with the human body. Polylactic acid, a member of the polyester family, is an ideal environmentally friendly macromolecular material extensively applied in the fields of medical treatment, public health, and agriculture. In fact, 5000 tons of PLA are manufactured by Zhejiang Hisun Biomaterials Co., Ltd. (China) annually. BMP was first discovered by Urist in 1963, and BMP-2 has been applied in spinal fusions to induce osteogenesis. In clinical practice, BMP-2 functions efficiently and safely in bone tissue engineering.

As the three compounds do not interfere with normal physiological activities, HA/PLGA/BMP-2 composite biomaterials were used in this study for possible implantation (Lin et al., 2010; Zheng et al., 2010), which has rarely been reported. Rabbit femurs, a well-established model of bone tissue defects (Fu et al., 2008; Wu et al., 2010), were implanted with the composite biomaterials in this study.

Xenogeneic cancellous bone-calcium alginate PLGA-BMP-2 composite scaffolds and chitosan/PLGA tissue-engineered nerve grafts have been widely applied in animal experiments, and have demonstrated satisfactory levels of compatibility and repair (Schopper et al., 2008; Hannink et al., 2013; Kleinschmidt et al., 2013). Although another rapid prototyping biomaterial, PLGA/tricalcium phosphate scaffold, is also histocompatible, it does not work as effectively as BMP in bone repair, because BMP efficiently induces the activation of osteogenesis (Kobayashi and Sakamoto, 2009; Thorey et al., 2011).

In summary, three groups of animals were implanted with biomaterials (HA/PLGA/BMP-2) composed of three different proportions of the same three constituents (HA, PLGA, and BMP-2), and the effects of these biomaterials were examined at two time points. The well-maintained growth

status observed indicates that it is feasible for the material, considered secure and histocompatible, to be applied in bone tissue engineering.

Conflicts of interest

The authors declare no conflict of interest.

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