Biomedicine & Pharmacotherapy 98 (2018) 325–332

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Exogenous VEGF introduced by bioceramic composite materials promotes the restoration of bone defect in rabbits

Hedong Yu[a], Xiantao Zeng[b], Cai Deng[a], Congyu Shi[a], Jun Ai[a], Weidong Leng[a,*]

[a] Department of Stomatology, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei, 442000, China
[b] Center for Evidence-Based and Translational Medicine, Zhongnan Hospital of Wuhan University, Wuhan, Hubei, 430071, China

ARTICLE INFO

*Keywords:*
VEGF
Polyether ketone
Bioceramic composite material
Bone defect
Bone graft
Bone repair

ABSTRACT

This study aimed to investigate the effect of exogenous vascular endothelial growth factor (VEGF) introduced by bioceramic composite materials on jawbone defect. Rabbits were randomly divided into four groups: control, sham, model, and stent. In the model group, holes of jawbone defect were created through surgery. In the stent group, rabbits with jawbone defect were treated with polyether ketone (PEK)/biphasic bioceramic ((PEK-BBC)) composite materials encapsulating VEGF. At 4, 8, and 16 weeks post-operation, HE and Van Gieson staining of jawbones were performed to characterize the repair status of the bone defect. For all time intervals, we found intact bone structures in the control and sham groups and there was no improvement in the bone defect position in the model group. However, in the stent group, we excitingly observed the growth of many osteocytes in the margin of stents at 8 and 16 weeks. RT-PCR, western blot, and immunofluorescence analysis were conducted to investigate the VEGF expression at 4, 8, and 16 weeks post-operation. At 8 weeks, the level of VEGF in the model group was sharply downregulated as compared with the control group ($P < .05$) and interestingly, the stent group had a much higher level of VEGF than the model group ($P < .05$). At 16 weeks, the VEGF expression in the model group was further reduced comparing to the control group ($P < .05$), which was also elevated to a relative high level by the stent treatment ($P < .05$). As for the sham group, the VEGF level was stable without any difference from the control group at all time intervals. Therefore, exogenous VEGF introduced by bioceramic composite materials promoted the restoration of bone defect in rabbits.

## 1. Introduction

Bone defect is a clinically common disease and it is a big challenge in bone repair treatment. Although bone autograft and allograft are known as common treatments, both of them have their own shortcomings.

In recent years, biological tissue engineering is utilized more and more popularly to achieve the reconstruction of bone defect. Tissue engineering materials and biological factors are the major parts of biological tissue engineering. Calcium phosphate-like biological calcium sulfate, ceramics, and polymers are some of familiar tissue engineering materials [1–3]. Thereinto, biological ceramics exhibits outstanding advantages such as biocompatibility, strong affinity to bodies, anti-thrombosis, erosion resistance, excellent mechanics, and physicochemical stability [4]. Polyether ketone (PEK) has been widely employed as bone repair material for its biocompatibility, similar elasticity to cortex, and high temperature, chemical, and radiation resistance [5–7].

Cytokines play vital roles in the bone reconstruction [8]. Vascular endothelial growth factor (VEGF) is a kind of cytokine that influences vascular endothelial cells at a manner of high specificity [9]. During the process of cartilage growing to bone, VEGF coordinates the relationships among chondrocytes succession, angiogenesis, osteoclast absorption, and osteogenesis [10]. Moreover, VEGF is able to facilitate the differentiation of osteoblasts, which will contribute a lot to the bone defect repair [10]. During fracture healing and vascular remodeling processes, VEGF expression is upregulated [11]. Furthermore, its upregulation is associated with the revascularization at the fracture end and promotes the proliferation of vascular endothelial cells [11].

Therefore in this study, VEGF was encapsulated into PEK/biphasic bioceramic (PEK-BBC) composite material to repair the jaw defect. The remediation effect was investigated by using hematoxylin-eosin (HE) staining and Van Gieson staining at various time intervals after operation. Then RT-PCR, western blot, and immunofluorescent analysis were employed to determine the VEGF expression at various time intervals after operation. This study might lay a foundation for the further

* Corresponding author.
*E-mail addresses:* yuhedong85@163.com (H. Yu), zengxiantao01@126.com (X. Zeng), deng_cai77@163.com (C. Deng), shicyu_ss@163.com (C. Shi), aj_aijun@163.com (J. Ai), lengweidong68@163.com (W. Leng).

https://doi.org/10.1016/j.biopha.2017.12.075
Received 17 September 2017; Received in revised form 15 December 2017; Accepted 15 December 2017

clinical application of PEK-BBC composite material encapsulating VEGF.

## 2. Materials and methods

### 2.1. Materials

SYBR Green qPCR SuperMix and Trizol were bought from Invitrogen (Carlsbad, CA, USA). Rabbit anti-VEGF and anti-β-actin polyclonal antibodies were purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H + L), RIPA buffer, and BCA kit were obtained from Beyotime Biotechnology (Jiangsu, China). 4,6-Diamidino-2-phenylindole (DAPI) kit was from Keygentec (KGA215-50, Jiangsu, China).

### 2.2. Animals

Twenty four New Zealand white rabbits were obtained from animal center of Nanchang University. These rabbits were raised with free access to water and food. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Hubei University of Medicine, China and was in accordance with the guidelines established by the Chinese Council of Animal Care.

### 2.3. Preparation of PEK-BBC composite material encapsulating VEGF

PEK-BBC composite material with double channels was prepared according to our previous report [12]. RADA16 polypeptide (Ac-RADARADARADARADA-$NH_2$) was synthesized and purified by Shanghai Bootech BioScience &Technology Co., Ltd., China. RADA16 polypeptide and VEGF were mixed with deionized water at 1% (w/v) and 100 g/L concentrations, respectively. The PEK-BBC composite material was immersed in the polypeptide hydrogel. When the material surface was completely wetted by the hydrogel, it was taken out and placed at 37 °C and aseptic condition for 0.5 h to solidify the hydrogel to fabricate the PEK-BBC composite material encapsulating VEGF.

### 2.4. Jaw defect model

Skin in the inferior margin of diaphysis molars of rabbit jawbone was disinfected with iodine tincture. Then the skin was cut open laterally and blunt dissection of the muscle was carried out to fully expose the groove part of molars. After crucial incision, periosteum was detached with a periosteal detacher to reveal the lateral cortical bone in the inferior margin of molars. The bone was grinded with a dental grinder from the middle of the inferior margin of molars. Gradually, the surrounding bone was also worn. Then we removed the tooth root and reserved the medial cortical bone. The bone defect was a square hole with 12 mm × 10 mm × 2 mm (length × width × depth). This coloboma was repeatedly flushed with abundant sterile normal saline to clear away any sundries such as bone debris. The models were built in the leftward positions (ipsilateral). Blank control group was set and bone graft materials were filled according to beforehand experimental design. After the restoration of the periosteum, the wounds were sutured layer by layer. During the suturing process, 80 thousand units of benzylpenicillin sodium were intramuscularly injected to prevent infection.

### 2.5. Animals grouping

Twenty four rabbits were randomly divided into four groups (n = 6): control, model, stent, and sham. Normal rabbits served as the control group. The model group was comprised of the rabbits with jaw defects. In the stent group, the jaw defects were repaired by (PEK-BBC) composite material containing VEGF. The sham group was composed of the rabbits suffering from the surgery but without repair treatments. Rabbits in all groups were euthanized at 4, 8, and 16 weeks post-operation respectively and jawbone samples were collected.

### 2.6. HE and Van Gieson staining

Jawbone samples with suitable size were washed with phosphate buffer solution (PBS) and fixed in 10% neutral formaldehyde buffer at room temperature for 1 h. Then the samples were decalcified in 5% hydrochloric acid for 3–4 days. After paraffin embedding, they were cut into slices. Subsequently, these slices were stained with HE and Van Gieson respectively. As to HE staining, briefly, the paraffin sections were dewaxed and hydrated. These slices were immersed in hematoxylin solution for 5 min and then washed with running water. After stained with eosin for 3 min, they were washed again with running water and dehydrated. The slices were mounted with neutral resin. As to Van Gieson staining, in brief, the paraffin sections were routinely dewaxed and stained in Weigert hematoxylin solution for 10–20 min. After washed with water for 5–10 min, they were incubated in Van Gieson solution for 2 min. After rapid differentiation by 95% ethanol for several seconds and dehydrated with ethanol and xylene, the slices were mounted. Finally, the slices were submitted to a light microscope (CKX41, Olympus, Japan) for observing the pathological change of the jawbones. The histological results were scored by determining mean optical density in three visual fields by ImagePro plus 6.0 software and expressed as lesion percent (%).

### 2.7. RT-PCR

Total RNA from the jawbones was extracted by Trizol according to the manufacture's instruction manual. RNA purity was determined. Then RNA was reversely transcribed and amplified with one step RT-PCR kit. Primers as demonstrated in Table 1 were added into a PCR reaction system (25 μl). The PCR parameters were set as follows: Denaturation for 45 s at 94 °C, annealing for 45 s at 59 °C, elongation for 60 s at 72 °C, and 35 circles. The amplification products (5 μl) were loaded onto 2% (w/v) agarose gel to conduct eletrophoresis. Electrophoresis strips were analyzed on a gel imaging system (ChemiDoc™ XRS, Bio-Rad, USA). β-Actin served as internal control.

### 2.8. Western blot

Jawbone samples were grinded in liquid nitrogen and then lysed in RIPA buffer for 30 min. The lysate was centrifuged at 4 °C and 10,000 rpm for 10 min. The supernatant was carefully collected to obtain total protein. Protein content was analyzed with a BCA kit. Then the protein was denatured, quantified, and loaded to perform SDS-PAGE electrophoresis for 1–2 h. Proteins were transferred to a membrane by a method of wet transfer for 30–50 min. The membrane was incubated with primary antibody buffer (rabbit anti-VEGF and anti-β-actin antibodies, both dilution 1:100) overnight at 4 °C. Then it was washed and incubated with secondary antibody buffer (dilution 1:100) for 1–2 h at room temperature. After rinse again, enhanced chemiluminescent (ECL) solution was dropped onto the membrane. Subsequently, the membrane was exposed on a gel imaging system (ChemiDoc™ XRS, Bio-Rad, USA). Gray values of proteins strips were analyzed with a Quantity one software (Bio-Rad, USA).

**Table 1**
Primers for RT-PCR.

| Gene | Primer (5′-3′) |
|---|---|
| VEGF | For: CCTGGAAGTCTACGAACGC<br>Rev: AGCAACCCGCACATCTCA |
| β-actin | For: ACTCTTCCAGCCTTCCTTC<br>Rev: ATCTCCTTCTGCATCCTGTC |