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Construction of Dual Gene-activated Matrix Via Coaxial Electrospinning and Its Application in Periodontal Tissue Regeneration

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ABSTRACT

Objective: This study aimed to construct a dual gene-activated matrix (GAM) via coaxial electrospinning and to investigate its application in periodontal tissue regeneration.

Methods: Coaxial electrospinning was used to encapsulate polyethylenimine (PEI)/ osteoprotegerin plasmid (p-OPG) cores in poly (lactic-co-glycolic acid) (PLGA) to form a dual GAM with a core-shell structure. PEI-PLGA core–shell nanoscaffolds were also prepared as a control. The average fiber diameters, mechanical properties, cell toxicities, p-OPG release behaviors, and OPG protein expressions of the prepared materials were compared to determine the utility of the scaffolds. Mouse experiments were used to study the effects of the dual GAM scaffolds on osteogenesis rates and their value in periodontal tissue regeneration.

Results: There were no significant differences between the mean diameters ($P > 0.05$) and mechanical properties ($P > 0.05$) of the fibers in each sample. No p-OPG was released from the control sample, whereas the amount of p-OPG released from the research sample increased over time ($P < 0.05$). The optical densities of both samples increased over time and there was no significant difference between them ($P > 0.05$). Over time, the transient transfection efficiency of the research sample gradually decreased and the OPG protein expression gradually increased. This was significantly different from the results for the control sample ($P < 0.05$). Finally, the bone formation rate in the research sample was higher than that in the control sample ($P < 0.05$).

Conclusion: Coaxial electrospinning can be used to construct dual GAMs. A PEI/p-OPG-PLGA core–shell GAM scaffold was prepared and cultured with periodontal ligament stem cells. There was very little cytotoxicity and the OPG plasmid was slowly and continuously released, transfected to the target cells, and continuously expressed. In mouse experiments, the PEI/p-OPG-PLGA core–shell GAM scaffolds promoted bone growth, indicating that they have considerable potential for periodontal tissue regeneration.

KEYWORDS: Coaxial electrospinning; Dual gene-activated matrix; Periodontal tissue regeneration; Osteoprotegerin

INTRODUCTION

Periodontal disease is a common illness that is characterized by chronic inflammation of the periodontal soft tissue. Current treatments typically involve the removal of infected tissue and the use of biofilms to guide regeneration. The goal of periodontal disease treatment is to control inflammation while promoting periodontal tissue regeneration and new attachments. Periodontal bone grafting, Guided tissue regeneration, periodontal tissue engineering and other technologies have brought new hope for the treatment of periodontal disease and the repair of periodontal defects, and have become a hot spot in periodontal tissue reconstruction research in recent years [1].

With advancements in tissue engineering, gene-activated matrices (GAMs) have been developed, which provide a local gene-release system by combining biological materials and plasmid vectors [2]. In contrast to direct supplementation of exogenous growth factors, GAM scaffolds provide a longer period of related gene expression. However, cytokines have not been integrated into bio-scaffold materials effectively to achieve slow and sustained release. Hence, the preparation of GAM scaffolds has become a popular research topic [3,4].

Coaxial electrospinning is a versatile fiber-manufacturing technique. Scaffold materials prepared using this technique can minimize the natural morphology of the extracellular matrix and they typically have small diameters and large specific surface areas [5]. However, few studies have used coaxial electrospinning to prepare GAM scaffolds. Therefore, this study aims to investigate this approach. Moreover, the prepared GAMs are applied to periodontal tissue regeneration to provide guidance for future practical applications, which is rarely discussed in the literature.

MATERIALS AND METHODS

Methods

Construction of the GAM scaffolds with core–shell structure: Polyethyleneimine (PEI) and Osteoprotegerin (OPG) plasmid microspheres were encapsulated in poly (lactic-co-glycolic acid (PLGA) copolymer shells to form PEI/p-OPG-PLGA scaffolds [6].

First, the "shell" solution was prepared by dissolving 0.75 g of PLGA in an appropriate volume of 12% (w/w) hexafluoroisopropanol (HFIP) solution under magnetic stirring. Second, separate "core" solutions were prepared for the research and control samples. For the research samples, PEI/p-DNA with a +/- charge ratio of 10 was dissolved in tris (hydroxymethyl) aminomethane–ethylenediaminetetraacetic acid (TE) buffer (0.1 × TE, pH = 7.5) to obtain a 500 μg/mL solution. Then, 1 mL of plasmid DNA solution and 750 μL of 99% pure PEI solution were added and ultrasonically stirred until combined. The mixture was left to rest for 30 min as the PEI/p-OPG-PLGA scaffolds developed. For the control samples, 750 μL of PEI was dissolved in 1 mL of TE buffer. Then, the mixture was stirred and left to rest as the PEI-PLGA scaffolds developed. The reagent concentrations and resting time were the same as those for the research sample [7].

Next, the prepared solutions were used for coaxial electrospinning. The "core" and "shell" solutions were delivered to the electrospinning machine using 5 mL and 10 mL syringes, respectively. The syringes were wrapped in aluminum foil to protect the contents from light. The electrospinning speed of the core and shell layers were 0.2mL/h and 10mL/h, respectively. Electrospinning was conducted at a voltage of 12 kW and a receiving distance of 15 cm.

In vitro transfection of periodontal ligament stem cells using PEI/p-OPG-PLGA GAM scaffold: Human periodontal ligament stem cells (hPDLSCs) and murine mononuclear macrophage leukemia cells (RAW264.7) were purchased from the Shanghai Institute of Chinese Academy of Sciences. The cell sourcing and other relevant ethical protocols, are explicitly addressed to the ethical considerations. The experimental procedures involving animal experiments or tissue samples in this study were conducted with the approval of the Ethics Committee of Tianjin Stomatological Hospital. The cell culture medium consisted of 10% fetal bovine serum, 1% penicillin-streptomycin mixture (double antibody), and Dulbecco's modified Eagle medium (DMEM). The hPDLSCs and RAW264.7 cells were cultured in a cell incubator at 37 °C and 5% CO_2. The medium was replaced every 2 days [8].

Cytotoxicity tests were conducted using MTT assays. Circular samples (1 cm diameter) of the membranes were sterilized with alcohol, rinsed with phosphate-buffered saline (PBS), placed in 24-well plates, and seeded with hPDLSCs at a density of 5×10^4 cells/mL. The cells were cultured using the method described above. After 1, 3, 5, and 7 d, 100 μL of thiazolyl blue was added to each well and the cells were cultured for another 4 h. The cells were cultured using the method described above. After 1, 3, 5, and 7 d, 100 μL of thiazolyl blue was added to each well and the cells were cultured for another 4 h. Then, the culture medium was poured out, 1 mL of dimethyl sulfoxide (DMSO) was added to each well, and the plate was shaken gently for 10 min. Then, a microplate reader was used to measure the optical density of each well at a wavelength of 490 nm. The average value was used to determine the cell viability.

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Observations

Average diameters of the electrospun fibers: Square samples (5 × 5 mm) of the membranes were adhered to the sample stage using conductive glue. The nanofiber morphology was observed by scanning electron microscopy and the accelerating voltage was 5 kV. For each sample, 60 fibers were randomly selected for analysis and Image-Pro was used to measure their diameters and to calculate the average value [9].

Mechanical properties of the membranes: Samples (1 × 6 cm) of the membranes 80–120 μm thick were prepared. A universal testing machine was used to test the Laber strength and elasticity of the membranes at a speed of 1 mm/min, the elastic modulus, and the elongation at break [10]. The tests were conducted at room temperature.

Sustained-release performance: Circular samples (10 mm diameter) of the membranes were sterilized with alcohol, rinsed with PBS, and seeded at a density of 5×10^4 cells/well. Then, the samples were added to polyethylene (PE) tubes with 2 mL of PBS and cultured in a shaking incubator. The eluate was collected after 1, 5, 19, 15, 20, 25, and 30 d and stored at -20 °C until the end of the experiment. The released plasmids were quantitatively analyzed using Pico-Green and then a microplate reader was used to measure the optical density at 530 nm.

***Cytotoxicity*:** The cytotoxicity of the electrospun membranes were determined using MTT assays. (The method was described in Section *In vitro transfection of periodontal ligament stem cells using PEI/p-OPG-PLGA GAM scaffold*)

***Transient transfection efficiency and p-OPG expression in the GAM cells*:** To determine the transient transfection efficiency, circular samples (10 mm diameter) of the membranes were sterilized with alcohol and rinsed with PBS. Then, the samples were added to PE tubes with 2 mL of PBS, which was replaced every 3 d as the samples were incubated. After 1, 5, 10, 15, 25, and 30 d, the membranes were removed and placed in a 24-well plate for flow cytometry analysis. The cells were observed using a laser confocal microscope and the transfection rate was calculated using flow cytometry counting 10000 cells [11,12].

To determine the p-OPG expression of the cells, circular samples (10 mm diameter) of the membranes were sterilized with alcohol, rinsed with PBS, placed in a 24-well plate, and seeded with hPDLSCs at a density of 5×10^4 cells/mL. After 1, 3, 7, 14, 21, and 30 d, the mRNA expression of OPG was observed and the protein expression level was determined *via* western blotting.

***Regeneration of extreme skull defects*:** A mouse model of extreme tooth defects was used to conduct membrane placement experiments [13]. Four mice were randomly selected as the research group and treated with PEI/p-OPG-PLGA core–shell GAM scaffolds. Four mice were used as the control group and they were treated with PEI-PLGA scaffolds. After 8 w of continuous feeding, all of the mice remained in good condition and the state of the tooth defects in the two groups were compared.

Statistical Methods

The measurement data were represented by ($\bar{x} \pm s$) and the t-test was used. The count data were represented by n (%) and the $\chi 2$ test was used.

Statistical Analysis

The data were analysed by SPSS version 23.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables with normal distribution were presents as mean ± standard deviation (SD); non-normal variables were reported as median (interquartile range). Mean of two continuous normally distributed variables were compared by independent samples Student's test. A value of $P<0.05$ was considered significant.

RESULTS

Average Diameters of the Electrospun Fibers

There was no notable difference between the mean diameters of the fibers in the research and control samples ($P > 0.05$), as shown in **Figure 1**.

Mechanical Properties of the Membranes

There was no significant difference between the mechanical properties of the research and control samples ($P > 0.05$), as shown in **Figure 2**.

Sustained-release Performance

Over time, the amount of p-OPG released from the research sample increased. This was significantly different from the

control sample ($P < 0.05$), which was not loaded with p-OPG, meaning that no p-OPG was release. This indicates that the PEI/p-OPG-PLGA GAM scaffolds released the p-OPG sustainably. The results for both samples are shown in **Figure 3**.

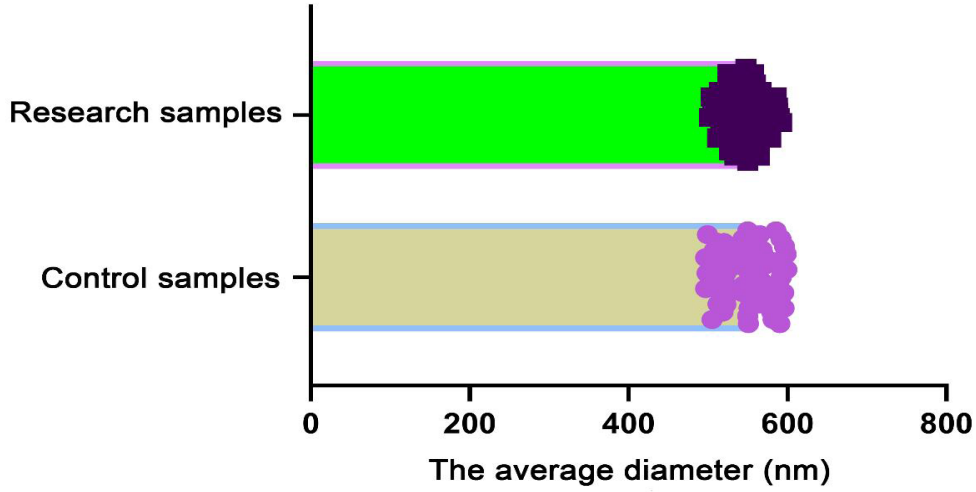

Figure 1: Average diameters of the fibers in the research and control samples.

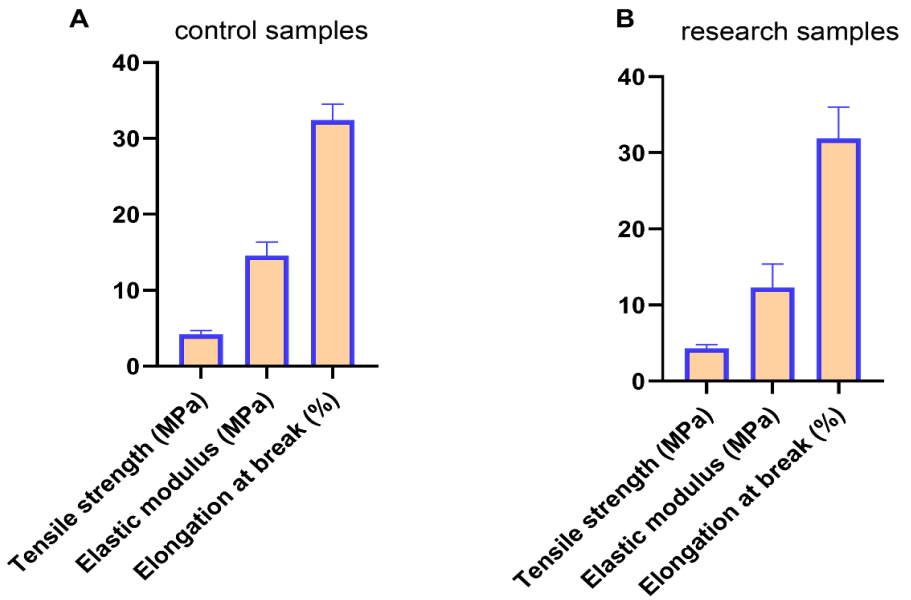

Figure 2: Mechanical properties of the (A) control and (B) research samples of the electrospun membranes.

Cytotoxicity

Over time, the optical densities of both samples increased. However, there was no notable difference between them ($P > 0.05$), which indicates that they had no negative effects on cell growth. The results are shown in **Figure 4**.

Transient Transfection Efficiency and p-OPG Expression with the GAM Scaffolds

Over time, the transient transfection efficiency of the research sample gradually decreased and the expression of the OPG protein gradually increased. The results were significantly different from those obtained for the control group ($P < 0.05$). This indicates that the PEI/p-OPG-PLGA scaffold promoted cell proliferation. The results are shown in **Figure 5**.

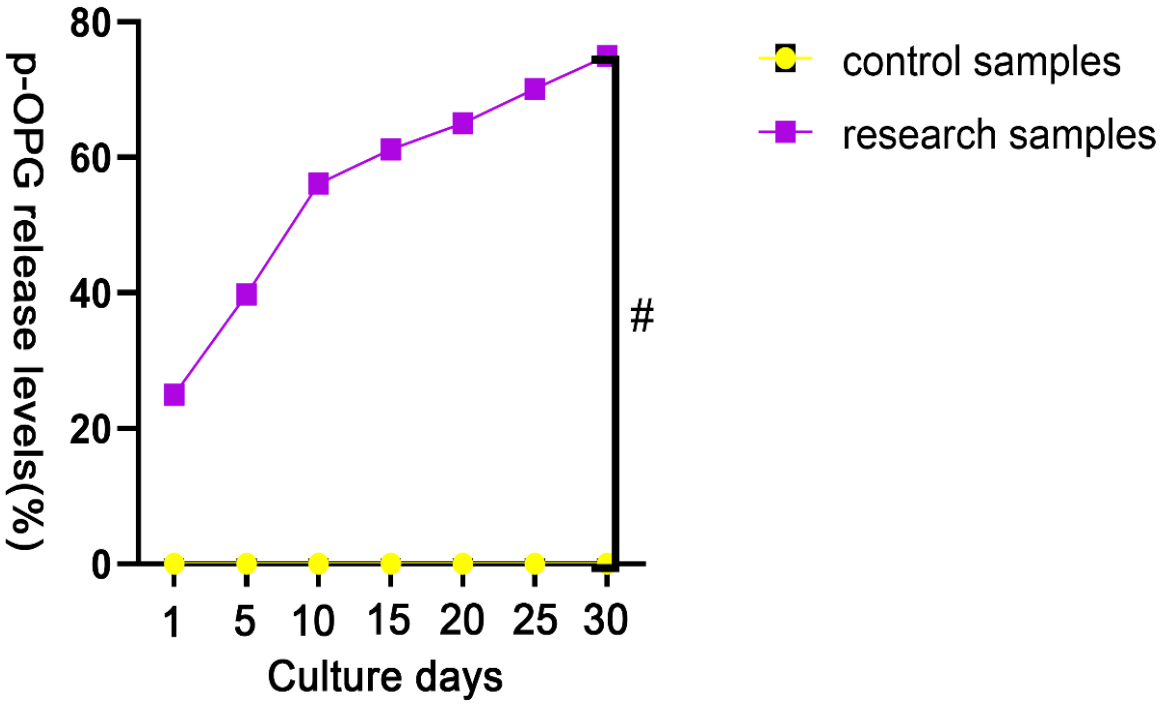

Figure 3: Sustained-release performance of the control and research samples. (Note: No p-OPG was detected for the control sample as the scaffolds were not loaded with p-OPG.).

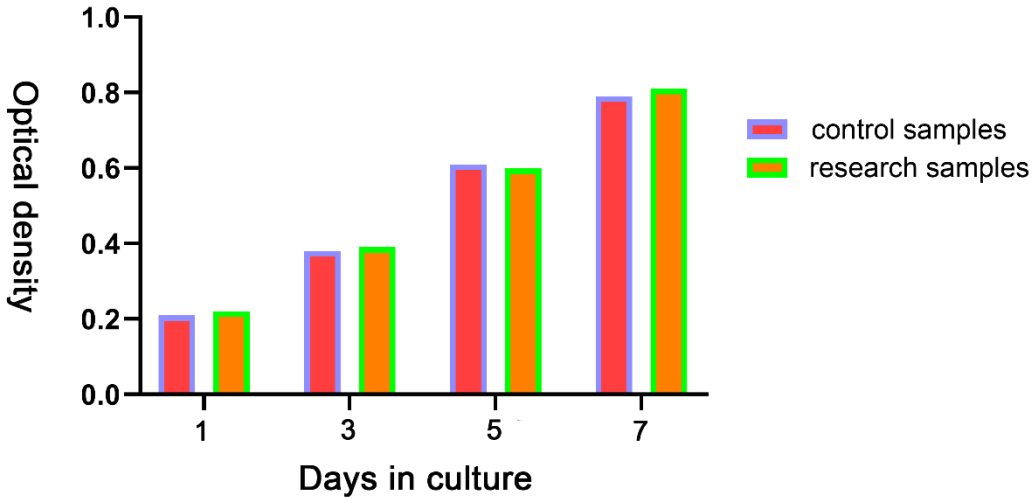

Figure 4: Cytotoxicity of the control and research samples.

Regeneration of Extreme Tooth Defects

The rate of osteogenesis in the mice in the research sample was higher than that in the mice in the control sample ($P < 0.05$), as shown in **Figure 6**. The results indicate that the PEI/p-OPG-PLGA GAM scaffold promoted the regeneration of extreme defects better than the control.

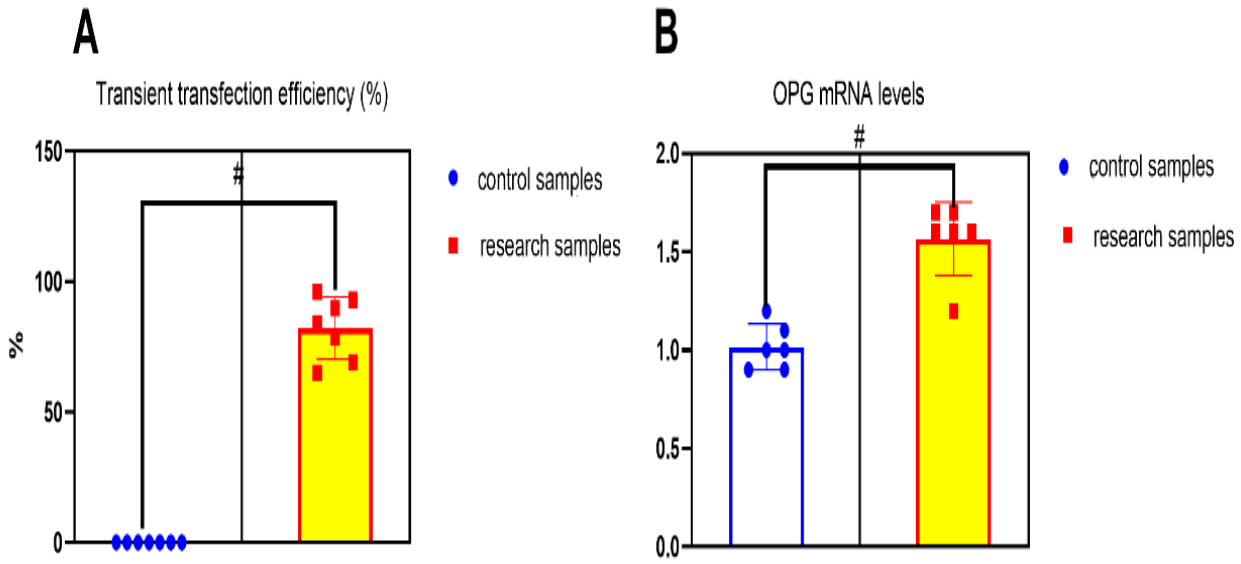

Figure 5: (A) Transient transfection efficiency and (B) p-OPG expression in GAM cells in the control and research samples. (Note: The scaffolds in the control group were not loaded with p-OPG. Therefore, the transient transfection efficiency was not detected.)

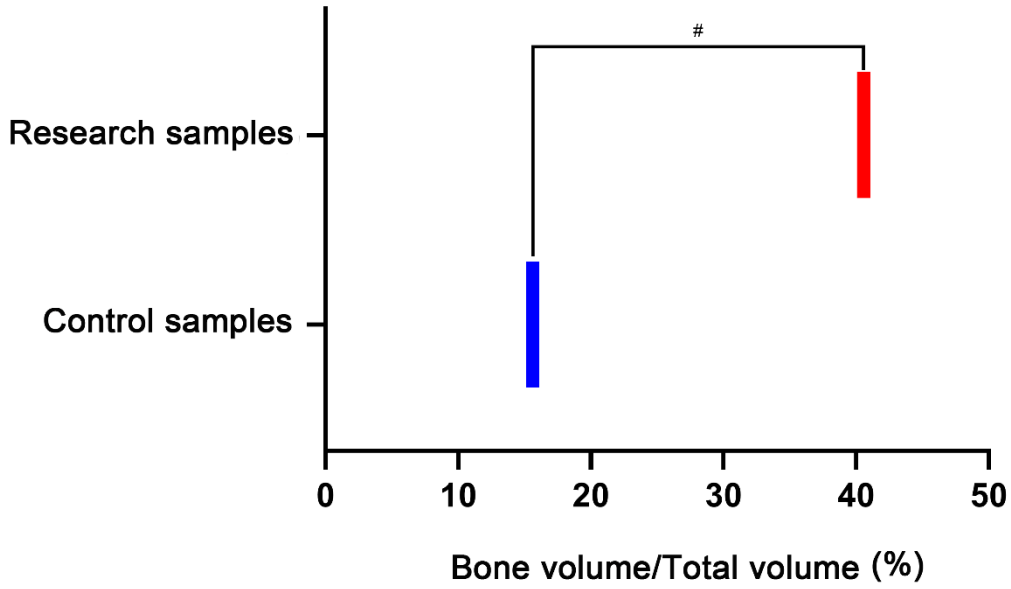

Figure 6: Restoration of the extreme tooth defects in the mice in the research and control groups.

DISCUSSION

As progress has been made in the medical field, the demand for periodontal tissue regeneration has increased. Hence, there is interest in the use of tissue engineering to repair the structures and functions of defective dental tissues. Well-prepared tissue-engineering scaffolds are a vital component of tissue-engineering technology [14]. Previous studies have shown that tissue regeneration relies on cytokines to regulate cellular variation, which positively affects the development of special tissue structures. Therefore, the preparation of biologically active scaffolds has become a popular research topic. Bioactive scaffolds provide mechanical support and promote the

regeneration of surrounding tissues via the localized release of growth factors [15,16]. Scaffolds prepared via coaxial electrospinning have high fiber contents, large specific surface areas, and interconnected structures. Moreover, they are porous three-dimensional structures that can establish a microenvironment similar to that of the cells in the body [17].

In this study, a PEI/p-OPG-PLGA core–shell scaffold was prepared. OPG particles were placed in the core layer, which isolated them from toxic solvents, preserved their biological activity, and facilitated the slow release of the target gene [18,19]. GAMs have good mechanical properties, which can effectively resist the mechanical forces caused by the surrounding tissues during healing; hence, they are convenient for practical operations. In this study, there was no significant difference between the diameters of the fibers with and without p-OPG, which were both on the nanoscale. Moreover, both materials were nontoxic to cells, which indicates that the PEI/p-OPG-PLGA core was prepared successfully using coaxial electrospinning technology. The mechanical properties and cytotoxicity of the core-shell GAM scaffolds can satisfy the actual healing requirements.

The application of a dual GAM requires the target factor to be delivered to the seed cells to promote the recovery of the defective tissue. Therefore, the loaded factors must be released from the membrane at a uniform and sustained rate [20]. In this study, p-OPG was wrapped with PEI in the PEI/p-OPG-PLGA core–shell scaffold so that it was slowly released as the PLGA degraded [21].

In the mouse experiments, the PEI/p-OPG-PLGA core–shell GAM scaffold significantly increased the bone formation rate compared to the PEI-PLGA scaffold, which indicated that p-OPG can promote bone-tissue regeneration. Dual GAM therapy has been developed for applications in gene activation chemistry, where tissue regeneration is promoted through the following mechanisms. First, auxin effectively binds to receptors in the plasma membrane and cytoplasm, which forms an auxin–receptor complex [22]. Second, the complex stimulates the production of inositol triphosphate, opens calcium channels, and increases the level of cellular Ca2+. Third, Ca^{2+} replaces H^{+}, which causes protein phosphorylation by affecting the ATPase [23]. Fourth, the protein factors bind to the auxins to form protein–auxin complexes, move to the nucleus, and convert them into special mRNA, which causes tissue regeneration by generating various substances required for cell growth [24,25].

This was a preliminary study and there are many gaps where further research is required. Although the dual GAM promoted the recovery of defective dental tissue, it is not clear how it can promote the recovery of periodontal tissue through the proposed mechanism and approach. Furthermore, few studies have investigated the application of dual GAMs in periodontal tissue regeneration. Consequently, the results of this study cannot be compared with those of other works. Therefore, further research is required to obtain more accurate experimental data to support the conclusions and to provide a more reliable theoretical basis for related applications.

CONCLUSION

This study shows that a dual GAM scaffold can be constructed using coaxial electrospinning technology. PEI/p-OPG-PLGA core–shell GAM scaffolds were prepared and cultured with periodontal ligament stem cells, which showed that there was no cytotoxicity. Moreover, the OPG plasmid was slowly and sustainably released, transfected into the target cells, and produced sustained expression. Mouse experiments showed that PEI/p-OPG-PLGA core–shell GAM scaffolds could promote bone growth, which indicates that they have considerable potential for applications in periodontal tissue regeneration.

ETHICAL STATEMENT

All protocols in this study were approved by the Ethics Committee of Tianjin Stomatological Hospital, in compliance with the Guide for the core and use of laboratory Animals published by the NIH. All the specimens were coded and handled according to the ethical guidelines described in the Code for Proper Secondary Use of Human Tissue in the Netherlands of the Dutch Federation of Medical Scientific Societies as reviewed and approved by the LUMC ethical board.

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