The Use of Zein and *Shuanghuangbu* for Periodontal Tissue Engineering

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Abstract

Aim Tissue engineering is a promising area with a broad range of applications in the fields of regenerative medicine and human health. The emergence of periodontal tissue engineering for clinical treatment of periodontal disease has opened a new therapeutic avenue. The choice of scaffold is crucial. This study was conducted to prepare zein scaffold and explore the suitability of zein and *Shuanghuangbu* for periodontal tissue engineering.

Methodology A zein scaffold was made using the solvent casting/particulate leaching method with sodium chloride (NaCl) particles as the porogen. The physical properties of the zein scaffold were evaluated by observing its shape and determining its pore structure and porosity. Cytotoxicity testing of the scaffold was carried out *via in vitro* cell culture experiments, including a liquid extraction experiment and the direct contact assay. Also, the Chinese medicine *Shuanghuangbu*, as a growth factor, was diluted by scaffold extract into different concentrations. This *Shuanghuangbu*-scaffold extract was then added to periodontal ligament cells (PDLCs) in order to determine its effect on cell proliferation.

Results The zein scaffold displayed a sponge-like structure with a high porosity and sufficient thickness. The porosity and pore size of the zein scaffold can be controlled by changing the porogen particles dosage and size. The porosity was up to 64.1%–78.0%. The pores were well-distributed, interconnected, and porous. The toxicity of the zein scaffold was graded as 0–1. Furthermore, PDLCs displayed full stretching and vigorous growth under scanning electronic microscope (SEM). *Shuanghuangbu*-scaffold extract could reinforce proliferation activity of PDLCs compared to the control group, especially at 100 μg·mL^{-1} (*P*<0.01).

Conclusion A zein scaffold with high porosity, open pore wall structure, and good biocompatibility is conducive to the growth of PDLCs. Zein could be used as scaffold to repair periodontal tissue defects. Also*, Shuanghuangbu*-scaffold extract can enhance the proliferation activity of PDLCs. Altogether, these findings provide the basis for *in vivo* testing on animals.

Keywords zein, periodontal tissue engineering, scaffold, periodontal ligament cells, *Shuanghuangbu*

Introduction

Periodontal disease is the primary cause of periodontal tissue defects, often leading to tooth loss in adults. The functional decline of teeth seriously affects health and quality of life (You *et al.*, 2009). For a long time, researchers have been trying to find innovative methods to effectively increase new attachment formation on teeth or reconstruct periodontal supporting tissues in order to treat periodontal disease. Although there has been some progresses in the treatment of peri-odontal defects, the available approaches still need improvement for reconstructing periodontal tissues. In recent years, the emergence of periodontal tissue engineering technology has opened up a new approach to the clinical treatment of periodontal diseases (Chen and Yan, 2010). An important prerequisite for tissue engineering is to find the appropriate scaffold material. Collagen (Dawson *et al.*, 2008), PLLA (Montjovent *et al.*, 2008) and hydroxyapatite (Thein Han *et al.*, 2009) have been used as scaffolds in tissue engineering. However, there are still many disadvantages using these materials. Therefore, obtaining the desirable scaffold material remains a high priority.

Zein is a mixture of alcohol soluble proteins that constitute 50%–60% of total endosperm protein. Because zein generally lacks lysine, tryptophan, and other human essential amino acids，its edible and nutritional value is relatively low. However, zein has a unique solubility, heat resistance, film-formation, antimicrobial properties, and anti-oxidative properties. It can be used for preventing moisture, isolating oxygen, as an anti-UV agent, an anti-static agent, and so on. Due to these characteristics, it has been the focus of exploration and application in many research areas. Studies have shown that zein is an important resource for the production of edible biodegradable food packaging materials (Ku and Song, 2007). The good antimicrobial and film-forming properties of zein have also been used for the preservation of fruits and vegetables (Marcos *et al.*, 2007; Del Nobile *et al.*, 2009). Thus, zein is a promising material in biomedical applications (Liu *et al.*, 2005; Bai, 2007; Yao *et al.*, 2007). No research has been reported, however, concerning the application of zein scaffold in periodontal tissue engineering. In this study, we investigated the use of zein as a suitable scaffold material for periodontal tissue regeneration.

Materials and Methods

Reagents and instruments

Zein (Wuhan Hezhong Biochemical Manufacturing Co., Ltd., China), sodium chloride (NaCl) analytical reagent, and anhydrous ethanol analytical reagent were from commercial sources. Dulbecco's modified Eagle Medium (DMEM) and trypsin were from Gibco (Invitrogen, USA). Fetal bovine serum was from Sijiqing (Hangzhou, China). The scanning electron microscope was from HITACHI (S-3500N, Japan), and the inverted phase contrast microscope was an Olympus IMT-2 (Olympus, Japan). *Rhizoma coptidis, Radix scutellariae,* and *Rhizoma drynariae* were provided by the Dispensary of Traditional Chinese Medicine in the Fourth Hospital of the Hebei Medical University.

Preparation of zein scaffold

Zein was dissolved in 65% alcohol. NaCl was added to the zein solution, and the resulting mixture was heated with stirring until NaCl was evenly dispersed into the gel system. The zein gel was cast into a certain size in the vessel by incubating at 85℃ for 14–18 h. Subsequently, the zein gel was placed in deionized water at 37℃ and the water was changed every 4 hours. When no precipitation was detected *via* silver nitrate, leaching was allowed to continue for another 24 h. Finally, the gel was dried at 37℃ in a biochemical incubator, and porous zein scaffold was obtained. Under the same size of NaCl particle (180–250 μm), three different mass concentrations of NaCl (70%, 75%, and 80%) were prepared for zein scaffolds to measure the relationship between the concentration of NaCl and scaffold porosity, as described in the pycnometer method (Shi *et al.*, 2001). Meanwhile, under 80% mass concentration of NaCl, three different sizes of NaCl particles (180–250 μm, 250–300 μm, 300–425 μm) were used for the zein scaffold in order to observe the relationship between NaCl particles and scaffold pore size with the scanning electron microscopy.

Preparation of scaffold extract and liquid extraction experiment

Based on the equivalent of 3 cm^2 total surface area per 1 ml extracting medium, as per the criteria of GB/T16886.12-2005, scaffolds were transferred into DMEM medium with 10% FBS , to be placed into a 37℃/ 5% CO$_2$ incubator for 72 h, obtaining 100% scaffold extract. The 100% scaffold extract was followed by serial dilutions using DMEM with 10% FBS to a final concentration of 10%, 50%, and 100%.

An ambush mandibular third molar was selected to scrape the middle 1/3 of periodontal ligament root. The samples were grown in primary culture as described by the tissue method (Situ and Wu, 1996). The third passage cells were seeded in 96-well plates at $1 \times 10^3/100$ μL per well, and grown in the 37℃/ 5% CO$_2$ incubator for 24 h. Culture medium was removed and cells were randomly divided into three groups: experimental group (containing 10%, 50%, and 100% of scaffold extract), positive control group (containing DMEM with 0.64% phenol), and negative control group (containing DMEM alone). Thirty wells were randomized to the groups, with six wells each in the positive and negative control groups, respectively. The other 18 wells were randomly distributed to

10%, 50%, and 100% of scaffold extract, with six wells per concentration. All samples were cultured in the 37℃/ 5% CO_2 incubator for 24 h, 48 h, and 72 h. Cell viability was measured by MTT colorimetric assay, and relative growth rate (Relative Growth Rate, RGR) was calculated according to the formula of RGR = (absorbance A value of experimental group / absorbance A value of negative control group) × 100%, while the toxicity of the material was evaluated based on GB/T16175-2008, as shown in Table 1.

Table 1   Cell toxicity grade

grade	0	1	2	3	4
RGR/%	≥100	80–99	50–79	30–49	0–29

Direct contact assay

The zein scaffold was treated with ultraviolet (UV) for 1 h, and soaked in DMEM solution containing 20% FBS overnight. The fourth passage cells at the concentration of 3.5×10^4 mL^{-1} were inoculated into the scaffold. After eighteen days, a light microscope and scanning electron microscope were used to observe the cell morphology.

Shuanghuangbu preparation and Cell proliferation assay with *shuanghuangbu*-scaffold extracts

A mixture of *Rhizoma coptidis*, *Radix scutellariae*, and *Rhizoma drynariae* (in the proportion of 2 : 1 : 1) was soaked in water (8 times and 6 times, respectively), boiled twice and filtered, and the concentration of 1 g·mL^{-1} *Shuanghuangbu* decoction was made. Then the *Shuanghuangbu* decoction was filtered and diluted with 100% scaffold extract to final concentrations (10, 25, 50, 100, 150, 200, 500, and 1 000 μg·mL^{-1}), respectively. The pH of the final *shuanghuangbu*-scaffold extracts was adjusted to 7.0–7.1, then they were stored at 4℃.

Cells were collected and inoculated as described above, and then were randomly divided into DMEM group，scaffold extract group，and *Shuanghuangbu*-scaffold extracts group with different concentrations (10, 25, 50, 100, 150, 200, 500, and 1000 μg·mL^{-1}). Sixty wells were randomized to the groups, with six wells in the DMEM group and scaffold extract groups, respectively. The other 48 wells were distributed to the *Shuanghuangbu*-scaffold extracts group, with six wells per concentration. The cells were cultured for 24 h, 48 h, and

72 h, and then measured by MTT to compare the proliferation of each group. Experiments were repeated three times.

Statistical analysis

SPSS13.0 statistical software was used to conduct single-factor ANOVA analysis. $P<0.05$ was considered as the threshold for a statistically significant difference.

Results

Physical properties of zein scaffolds

The zein scaffold displayed a high porosity with a sponge-like structure, and the pores on the zein scaffold were distributed evenly. Their shape was variable, but mostly round and interconnected. Furthermore, the pore sizes were mostly similar, but scattered with relatively large ones (Figure 1).

Figure 1   The photo of zein scaffold

Zein scaffold has a sponge-like structure, with pores well-distributed.

With the same size of the porogens, zein scaffold porosity was closely related to the percentage of porogen. As the percentage of porogen increased, the porosity of scaffolds increased accordingly (Table 2).

Table 2   Relationship between the porosity of scaffolds and the percentage of porogen (mean ± SD)

Percentage/ %	*n*	Porosity/ %
70	30	64.1 ± 2 .7
75	30	70.5 ± 2.6
80	30	78.0 ± 2.2

Under the same amount of porogen, zein scaffolds showed the porous network structure. The sizes between pores and particles of porogen were clearly correlated. As the porogen particle diameter increased, the size of the pores also increased (Figure 2). In addition, a few microns
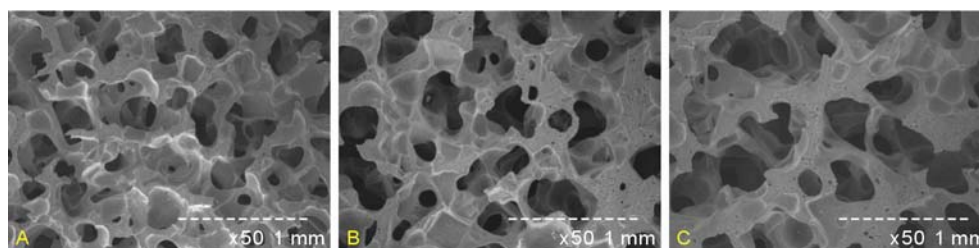
Figure 2   The size of porogen were (A) 180–250 μm, (B) 250–300 μm, (C) 300–425 μm (SEM, × 50)

The size of scaffold pores increased when porogen particle diameter increased.
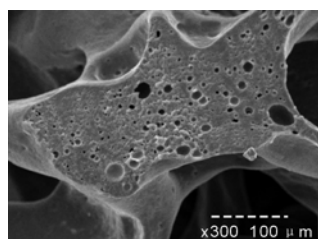

Figure 3   Microns pores observation (SEM, × 300)

Microns pores were scattered in scaffold walls.

to tens of microns-sized pores were distributed in scaffold walls, being beneficial by acting as conduits among pores, and increasing the roughness of pore walls (Figure 3).

Liquid extraction experiment

Zein scaffolds were biologically evaluated by cytotoxicity tests which were carried out based on liquid extraction experiment and direct contact assays. The results of liquid extraction experiment showed that for culturing 24 h, 48 h, and 72 h, the positive control and the negative control had a significant difference ($P<0.01$). The experimental group and negative control group showed no significant difference ($P>0.05$). Based on GB/T16175-2008, the cell toxicity of phenol was rated as 3–4 grades, but the cell toxicity of zein scaffolds was rated as the 0–1 grade (Table 3).

Direct contact assay

These cells displayed the elongated spindle-like shape and were grown along the pores under observation with the inverted phase contrast microscope. More periodontal ligament cells showed a tight attachment to zein scaffolds under scanning electron microscope. These cells were fully stretched, star-shaped, or spindle-shaped, extending several pseudopodia-like protrusions along the edge of the pore, and closely linked with the surrounding cells (Figure 4).

Table 3   Toxicity grade for zein scaffold (mean ± SD)

Group	24 h			48 h			72 h		
	A value	RGR	Grade	A value	RGR	Grade	A value	RGR	Grade
DMEM (negative)	0.441 ± 0.030	100	0	0.464 ± 0.021	100	0	0.537 ± 0.017	100	0
10％ extract	0.440 ± 0.023	100	1	0.467 ± 0.018	105	0	0.541 ± 0.017	100	0
50％ extract	0.446 ± 0.012	107	0	0.475 ± 0.012	111	0	0.540 ± 0.020	106	0
100％ extract	0.443 ± 0.018	100	0	0.472 ± 0.012	111	0	0.543 ± 0.019	108	0
Phenol (positive)	0.199 ± 0.013[a]	45	3	0.177 ± 0.012[a]	38	3	0.123 ± 0.012[a]	23	4

extracts *vs* negative control，*P*>0.05; positive control *vs* negative control，[a]*P*<0.01.
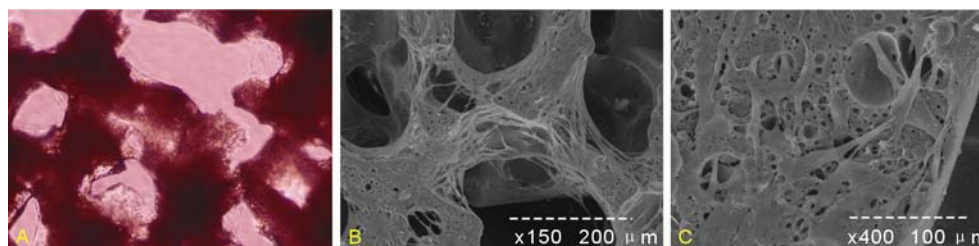

Figure 4   Periodontal ligament cells attached to zein scaffolds

A: inverted phase contrast microscope, × 100; B: SEM, × 150; C: SEM, × 400. PDLCs grew along the pores and tight attached to zein scaffolds.

Table 4 The effects of *shuanghuangbu*-scaffold extracts at various concentrations on the proliferation of periodontal ligament cells (mean ± SD)

Group	*Shuanghuangbu* concentrations/ μg·mL^{-1}	24 h	48 h	72 h
DMEM	0	0.336 ± 0.027	0.429 ± 0.029	0.453 ± 0.020
scaffold extract	0	0.336 ± 0.012	0.430 ± 0.012	0.456 ± 0.019
shuanghuangbu-scaffold	10	0.356 ± 0.031	0.441 ± 0.025	0.467 ± 0.021
extracts	25	0.362 ± 0.032[bc]	0.436 ± 0.042	0.473 ± 0.032
	50	0.360 ± 0.026[ac]	0.453 ± 0.024[ac]	0.477 ± 0.021[a]
	100	0.373 ± 0.029[bd]	0.458 ± 0.016[bd]	0.483 ± 0.019[bd]
	150	0.338 ± 0.013	0.444 ± 0.015	0.478 ± 0.018[ac]
	200	0.344 ± 0.023	0.443 ± 0.025	0.463 ± 0.018
	500	0.338 ± 0.014	0.436 ± 0.026	0.455 ± 0.019
	1000	0.337 ± 0.011	0.432 ± 0.015	0.454 ± 0.036

Scaffold extract *vs* DMEM，*P*＞0.05；*shuanghuangbu*-scaffold extracts *vs* scaffold extract，[a]*P*＜0.05，[b]*P*＜0.01；*shuanghuangbu*-scaffold extracts *vs* DMEM，[c]*P*＜0.05，[d]*P*＜0.01.

Cell proliferation assay with *Shuanghuangbu*-scaffold extracts

Results showed that, compared with DMEM group, the scaffold extract group had no significant difference in terms of PDLC growth (*P*>0.05). Compared with the DMEM group and the scaffold extract group, different concentrations of the *Shuanghuangbu*-scaffold extract could enhance cell proliferation differently, but 100 μg·mL^{-1} had statistically the most significant effect (*P*<0.01), Table 4.

Discussion

Periodontal tissue engineering techniques for generating scaffold material are an important topic in the field of dental research. In this paper, zein, a corn protein, was used as a scaffold material, and its suitability for tissue engineering was explored. We demonstrated that zein scaffold may be feasible for periodontal tissue engineering through liquid extraction experiments, the direct contact assay, and cell proliferation assay with *shuanghuangbu*-scaffold extracts. Also, these results led us to conclude that zein scaffold has a definite potential for biomedical applications.

Zein protein has been studied in many areas. For example, Dong (Dong *et al.*, 2004) successfully applied zein to human liver cells (HL-7702) and rodent fibroblasts (NH3T3). Heparin-loaded zein microsphere film and its degraded product had better biocompatibility when they were used

to culture human umbilical vein endothelial cells (Wang *et al.*, 2005). Gong (Gong *et al.*, 2006) further demonstrated that zein scaffold can promote rat bone marrow stromal cell adhesion, growth, and differentiation, suggesting that zein protein possesses good histocompatibility (Wang *et al.*, 2007). Here zein as a scaffold material was studied for periodontal tissue engineering. Results showed that the porosity of the zein scaffold was 64.1% – 78.0%, and increased as the percentage of the NaCl porogen increased. The scaffold diameter increased when the porogen particle size increased. Thus, one can prepare zein scaffolds with different porosity and pore size by adjusting the percentage and particle size of porogens. In addition, results from SEM showed that many micropores with the diameter of a few microns to a few tens of microns were distributed on the scaffold walls, improving the linkage between the porosities, and also enhancing the roughness of the scaffold surface, which is conducive to cell growth and the integration of cell organization (Anselme, 2000).

In order to verify if zein scaffold benefits cell growth, a toxic assay on cell growth was conducted. Periodontal ligament cells grown in primary culture was used in this study and two kinds of experiments including the liquid extraction experiment and the direct contact assay were carried out. Results showed that these scaffold extracts had no effect on the activity of PDLC proliferation and no toxic reaction either. PDLCs stretched out completely, displayed multiple pseudopodia-like protrusions trespassing the mcirohole surface, or along the

microhole wall on the scaffold materials, as observed under SEM in the direct contact assay. This indicates that the scaffold materials had good cell compatibility and no potential toxicity.

There are a number of ways to prepare the scaffolds (Gomes *et al.*, 2006; Petrie Aronin *et al.* 2008; Wei *et al.*, 2009). The solution casting/ particulate leaching method is the most popular one, because of the simple equipment, controllable pore size, and porosity. However, there are several shortcomings in this method, such as the less than 2 mm thickness of the scaffold caused by the particle deposition, the lower porous nature of inter-linking, and the organic solvent residues left that may affect cell growth. In order to overcome these shortcomings, some researchers have suggested approaches (Murphy *et al.*, 2002). We has modified some procedures by using the heating system during stirring, which creates a uniform distribution of porogen gelatinous, casting small settlement after the porogens, and creating more than a 2 mm frame thickness of the scaffold. In addition, water-bath heating was used to promote evaporation of alcohol in a relatively short period of time. Volatile elements during the overflow went through the channel and increased the inter-linking nature of the pore. Alcohol has been used because of its low toxicity. When used with heat, alcohol was evaporated and cell toxicity caused by the residual amount was low.

Periodontal tissue repair and reconstruction is closely related to periodontal ligament cell proliferation and differentiation. Whether periodontal tissue engineering can proceed smoothly or not lies in the activity of PDLC proliferation and differentiation. Some studies have shown that a variety of growth factors are involved in strengthening cell chemotaxis function (Zaman *et al.*, 1999)，but the effect of applying a single growth factor is not always good (Oates *et al.*, 1993). Thus, several growth factors must be combined in spite of those high prices. In recent years, studies have shown that the Chinese medicine *Shuanghuangbu* can prompt the proliferation, differentiation, and protein synthesis of PDLCs (Xu *et al.*, 2004; Xu *et al.*, 2009). *Shuanghuangbu* can obviously promote new bone and cementum formation, restrain the migration of junctional epithelium, and enhance the regeneration of periodontal tissue (Xu *et al.*, 2005)；*Shuanghuangbu* sustained-release preparation can significantly improve the clinical symptom of periodontitis and change the composition of subgingival microflora at disease sites to that at healthy sites (Xu *et al.*, 2004). Thus, we thought that applying *Shuanghuangbu* as a growth factor to periodontal tissue engineering may achieve a satisfactory effect. This research found that different concentrations of the *Shunghuangbu*-scaffold extract could enhance cell proliferation to various degrees, but 100 μg·mL^{-1} showed statistically the most significant effect. These findings provide an objective basis for consideration of zein-*shuanghuangbu*-PDLC complex for periodontal tissue regeneration in clinical applications.

Conclusions

We conclude that zein scaffold has high porosity, opened pore wall structure, and non-toxicity. The good biocompatibility is conducive to the growth of PDLCs. These results provide the evidence of zein's suitability as scaffold material for periodontal tissue engineering. Also, *Shuanghuangbu*-scaffold extract can enhance the proliferation activity of PDLCs. Future studies of *in vivo* testing on animals are required to further clarify the combination of *Shuanghuangbu*-zein scaffold-cell and how to repair periodontal defects.

Acknowledgrments

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