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Biocompatibility of a chitosan-based injectable thermosensitive hydrogel and its effects on dog periodontal tissue regeneration

Qiu Xia Ji[a,b,*], Jing Deng[a], Xiao Ming Xing[c], Chang Qing Yuan[a], Xin Bo Yu[a], Quan Chen Xu[a], Jin Yue[a]

[a] Department of Stomatology, The Affiliated Hospital of Medical College, Qingdao University, Qingdao, Shandong Province 266003, PR China
[b] Lab of Biochemistry and Marine Biomaterials, College of Marine Life Science, Ocean University of China, Qingdao, Shandong Province 266003, PR China
[c] Department of Pathology, The Affiliated Hospital of Medical College, Qingdao University, Qingdao, Shandong Province 266003, PR China

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ABSTRACT

We evaluated the biocompatibility of an injectable chitosan-based thermosensitive hydrogel, which was prepared from chitosan and quaternized chitosan (HTCC) containing α,β-GP (CS-HTCC/GP). The ability of the CS-HTCC/GP thermosensitive hydrogel to partake in periodontal tissue regeneration was investigated. The CS-HTCC/GP thermosensitive hydrogel was found to have no toxic effects and could promote alkaline phosphatase (ALP) activity in human periodontal ligament cells (HPDLCs) *in vitro*. An extract of the CS-HTCC/GP thermosensitive hydrogel had no obvious influence on the ultrastructure of HPDLCs. The CS-HTCC/GP solution was injected into the rumps of rats resulting in a non-specific inflammation reaction. CS-HTCC/GP thermosensitive hydrogel loading with basic fibroblast growth factor (CS-HTCC/GP-bFGF) more effectively enhanced new periodontal support tissues in dogs. All results indicated that the CS-HTCC/GP thermosensitive hydrogel exhibited excellent biocompatibility and had potential as an injectable local drug delivery vehicle and tissue-engineering scaffold for periodontal disease therapy.

1. Introduction

Dentistry is one of the earliest fields to have used artificial biomaterials for the treatment of diseased tissues. Golden teeth are representative examples of this, dating back more than 2000 years ago (Leeuwenburgh et al., 2008). Other early examples of biomaterials include wooden teeth and metallic dental implants (Langer & Peppas, 2003). Currently, a number of biomaterials are utilized in various dental procedures and treatments. The single most important factor that distinguishes a biomaterial from any other material is its ability to co-exist with tissues of the human body without causing an unacceptable degree of harm to that body (Williams, 2008). Accordingly, only after a biomedical material has been proven to be biocompatible can it provide further benefits for *in vivo* applications.

In recent years, more and more attention has focused on hydrogels, especially biodegradable and injectable *in situ* thermosensitive hydrogels, which could possibly be widely utilized in drug delivery and tissue regeneration (Quirynen, Teughels, & van Steenberghe, 2003; Roberts, 2002; Southard & Godowski, 1998; van Winkelhoff et al., 2000). Thermosensitive hydrogels can remain in a liquid state at room temperature for a long time and turn into a gel if heated to normal body temperature. It can be easily delivered, exhibit good retention at the application site, and display a controlled release of drugs. So, it can keep high level drug(s) in the gingival crevicular fluid (GCF) for the desired clinical effects on periodontal disease. Additionally, it has gained high patient acceptance and is a method of application acceptable to dentist's requests (Southard & Godowski, 1998). Therefore, *in situ* biodegradable thermosensitive hydrogels have been recognized as one of the most convenient and reliable methods to prevent periodontal disease.

Many biomaterials have been used for periodontal disease treatment since the 1970s but there is still lack of comprehensive data regarding evaluation of biocompatibility. In nature, chitosan is a well-known parvus polysaccharide that is prepared by the deacetylation of chitin, mainly obtained from crab and shrimp shells. Chitosan-based thermosensitivity has been widely investigated and utilized in drug delivery (Barreiro-Iglesias, Coronilla, Concheiro, & Alvarez-Lorenzo, 2005), cell encapsulation (Lagarce et al., 2005) and tissue engineering (Cho et al., 2004).

Periodontal diseases are infectious diseases that are characterized by the destruction of periodontium (the supporting tissue for teeth), including the periodontal ligament (PDL), cementum, alveolar bone and gingival (Seo et al., 2004). These are generally destructive, non-reversible conditions which can result in the formation of pockets between gingiva and tooth, gingival margin retraction and, ultimately, possible tooth loss (Tatakis & Kumar, 2005). Periodontal disease is already a serious public health

* Corresponding author. Tel.: +86 532 8291 1213; fax: +86 532 8291 1840.
E-mail address: jqx_1@163.com (Q.X. Ji).

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concern all over the world due to its high prevalence and serious symptoms. The final aim of periodontal therapy is to remove the bacterial deposits from the tooth surface and induce periodontal tissue regeneration.

In our preliminary study, a novel thermosensitive hydrogel was successfully designed and prepared. This hydrogel was based upon chitosan and its derivate, quaternized chitosan (HTCC) with α,β-glycerophosphate (α,β-GP), without any additional chemical cross-linkers as a local drug delivery system for periodontal therapy (Ji et al., 2009). The biocompatibilities of the CS-HTCC/GP thermosensitive hydrogel *in vivo* and *in vitro* were evaluated in accordance with ISO10993-1:1997 (1997) and GB/T 16886.1-2001 (2001). We investigated the response of primary cultured human periodontal ligament cells (HPDLCs) to the CS-HTCC/GP thermosensitive hydrogel and examined cell proliferation, alkaline phosphatase (ALP) activity and the morphology of HPDLCs *in vitro*. Assessment *in vivo* was done by injecting an aqueous preparation of the CS-HTCC/GP thermosensitive hydrogel intramuscularly into the rumps of rats and examining the histology. Additionally, dog models of periodontal defects were utilized. CS-HTCC/GP and that of loading with basic fibroblast growth factor (CS-HTCC/GP-bFGF) thermosensitive hydrogels were implanted into the periodontal defects to evaluate the regenerative activity.

2. Materials and methods

2.1. Materials

Chitosan (1080 kDa, deacetylation degree 86%) and glycidyltrimethylammonium chloride (GTMAC) were obtained from Dongying Guofeng Fine Chemical Co. Ltd. (Shandong, China). The α,β-glycerophosphate (α,β-GP) was provided by Kaiyuan Pharmaceutical & Chemical Co., Ltd. (Shanxi, China). Ornidazole (ONZ) was kindly donated by Xi'an Bodyguard Pharmaceutical Co. Ltd. (Batch No. 0706083; Shanxi, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ALP kits were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco (Carlsbad, CA, USA).

Dogs and Sprague–Dawley rats were used in this study and animal experiments were conducted according to the Animal Ethical Committee of Medical College, Qingdao University (Qingdao, China), which adhered to the policies and principles established by the Animal Welfare Act and the recommendations in the Guide for Care and Use of Laboratory Animals.

2.2. Preparation of CS-HTCC/GP thermosensitive hydrogel

N-[(2-hydroxy-3-trimethylammonium)propyl] (HTCC), a water-soluble chitosan derivative, was prepared by reacting chitosan with GTMAC. CS-HTCC/GP thermosensitive hydrogel was prepared by mixing 0.15 g chitosan sulfate (CS) and 0.03 g HTCC (2%, w/v) and progressively adding this mixture to 7 ml 0.1 M aqueous lactic acid (LA) solution at room temperature with stirring until it was completely dissolved. Following this, 2 ml H_2O was added to dilute the solution. The α,β-GP aqueous solution (50%, w/v) was prepared in deionized water. Both solutions were chilled in an ice bath for 15 min. The α,β-GP solution was added dropwise to the chitosan solution with stirring. The final concentration of α,β-GP was 8.33% and the solution obtained was stirred for 20 min

The CS/α,β-GP thermosensitive hydrogel was prepared by adding 0.18 g CS to 7 ml 0.1 M aqueous LA solution. The other steps were the same as for the CS-HTCC/GP thermosensitive hydrogel as described above.

2.3. Characterization of the CS-HTCC/GP thermosensitive hydrogel

The thermosensitivity of the hydrogel at 37 °C was determined by the test tube inverting method (Chung, Simmons, Gutowska, & Jeong, 2002). The samples were coated with platinum using ion sputter gold under vacuum and the surface was investigated by scanning electron microscopy (KYKY2800B; KYKY Technology Development Ltd., Beijing, China).

2.4. In vitro cytocompatibility study

2.4.1. Isolation and culture of HPDLCs

Primary HPDLCs were obtained from premolars that had been extracted from healthy patients (11–16 years old) for orthodontic reasons with a good oral health status (no evidence of gingivitis, periodontitis or caries). All patients gave informed consent before providing samples. Briefly, the human periodontal ligament tissues were obtained from the center of the tooth root surface with a surgical scalpel. The tissues were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin solution (5000 units/ml penicillin and 50 μg/ml streptomycin; Sigma Chemical Co.) at 37 °C/5% CO_2 in a humidified atmosphere with the medium changed every 2–3 days. When the HPDLCs cultured from the tissue fragments reached confluence, the cells were then trypsinized and split at a ratio of 1:2 with 0.25% trypsin solution. Cells at passages 3–5 were used for this study.

2.4.2. Preparation of the CS-HTCC/GP thermosensitive hydrogel extract

According to ISO standards, the ratio between the surface of the sample and the volume of the medium was 0.5 cm^2/ml (Camps & About, 2003). The CS-HTCC/GP thermosensitive hydrogel was soaked in culture medium at 37 °C for 24 h. The culture medium was then filtered through a 0.2 μm filter (2003). The extracts containing almost all the soluble compounds of the hydrogel in culture medium were obtained and diluted with DMEM containing 10% FBS to 50, 25 and 12.5% (v/v).

2.4.3. MTT assay

Cytotoxicity was measured by an MTT assay. Briefly, cells were plated into 96-well flat-bottom microplates (Costar, USA) at a density of approximately 3×10^4 cells/well in medium containing 10% (v/v) FBS. The medium was replaced after 24 h and the cells were divided into four different groups, which were treated with DMEM containing different concentrations of CS-HTCC/GP hydrogel extracts: group 1, 50% (v/v); group 2, 25% (v/v); group 3, 12.5% (v/v); and group 4, negative controls. After culturing for 1, 3 and 5 days, 200 μl of MTT solution [2 mg/ml diluted in phosphate-buffered saline (PBS)] was added to each well and incubated for 4 h. Subsequently, 200 μl of dimethyl sulfoxide (DMSO) was added to each well. The plates were then shaken until the crystals dissolved and the absorbance was determined at 490 nm with a microplate reader (Bio-Rad Model 550; Bio-Rad, Hercules, CA, USA). All reported values were the means of triplicate samples and this test was repeated twice.

2.4.4. Measurement of ALP activity

ALP activity in the cells was measured by p-nitrophenyl phosphate (p-NPP) substrate reactions using Sigma ALP assay reagents. After culturing for the indicated amount of time, cells were washed twice with PBS and incubated with 50 μl of 0.2% Triton X-100 for 20 min with gentle shaking. The cells were then incubated with 100 μl substrate (10 mM p-NPP, 1 mM $MgCl_2$) for 30 min at 37 °C. The reaction was stopped by adding 100 μl of 1 M NaOH. The p-nitrophenol formed was measured at 405 nm using a microplate

reader (Bio-Rad Model 550). Cell numbers were evaluated using a hemacytometer. ALP activity data were converted to units of ALP per 10^4 cells.

2.4.5. Morphological study of HPDLCs

The morphology of HPDLCs was observed by light microscopy and transmission electron microscopy (TEM). The ultrastructure of HPDLCs was analyzed in groups 1 and 4. The density of HPDLCs was adjusted to 5×10^4 cells/ml in culture medium containing 10% (v/v) FBS. After incubating at 37 °C for 72 h, HPDLCs were centrifuged for 15–20 min after adding 10 ml ice-cold Hank's buffered salt solution. The pellets were immediately fixed in cold 4% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 °C. The pellets were then dehydrated in graded acetone, passed through propylene oxide and embedded in Epon 812. 2 μm thick sections were cut, stained with toluidine blue sodium tetraborate and observed under a light microscope. Ultrathin sections were also obtained from the same specimens and stained with uranyl acetate and alkaline bismite subnitrate and then examined by TEM. TEM was performed using a JEM-1200EX electron microscope operating at 60 kV.

2.5. In vivo histocompatibility study

The *in vivo* histocompatibility test for the CS-HTCC/GP hydrogel was performed by injecting ice-cold aqueous CS-HTCC/GP into male Sprague–Dawley rats (200–220 g). After shaving and disinfection, 0.4 ml of the UV-sterilized CS-HTCC/GP aqueous solution was injected intramuscularly into the left and right back regions of the Sprague–Dawley rats. After 3 days, 1, 2, 4, 6 and 9 weeks, the rats were sacrificed and the surrounding tissue where the hydrogels were injected were isolated. The samples were fixed in 10% neutral buffered formalin and dehydrated through a graded ethanol series and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Tissue sections, 5 μm thick, were deparaffinized with xylene, stained with hematoxylin and eosin (HE) and studied using a light microscope.

Food and water were provided to animals *ad libitum* and no antibiotics were administered. All rats were closely observed and the care and handling of animals was performed with the approval of the Institutional Authority for Laboratory Animal Care.

2.6. Implantation of CS-HTCC/GP thermosensitive hydrogel in dog periodontal defect models

Four young adult male mongrel dogs (12 months old with a weight of 20.0 ± 2.0 kg) were purchased from the experimental animal center of Qingdao University Medical College (Shandong, China) and used after 2 weeks in quarantine. The animals were in good general health with no infections. Experimental defects (Class III) prepared in this study were based on an established model as previously described (Chen et al., 2006). The second and third premolars in each of the four dogs were selected for experimentation, so a total of 32 defects were obtained, and those defects were randomly allocated into three groups: group 1, CS-HTCC/GP hydrogel with bFGF (CS-HTCC/GP-bFGF); group 2, CS-HTCC/GP hydrogel; and group 3, negative controls. The CS-HTCC/GP and CS-HTCC/GP-bFGF thermosensitive hydrogels were sterilized and implanted into 10 teeth with defects respectively. The four dogs were euthanized 8 weeks post-surgery. Maxilla and mandibles were retrieved and stained *via* Mallory's trichrome staining methods. New bone formation (NB), new periodontal ligament regeneration (PDL) and new cementum formation (NC) were examined with a light microscope (Leica DMR). The heights of new bone formation, new cementum formation and new periodontal ligament regeneration were measured.

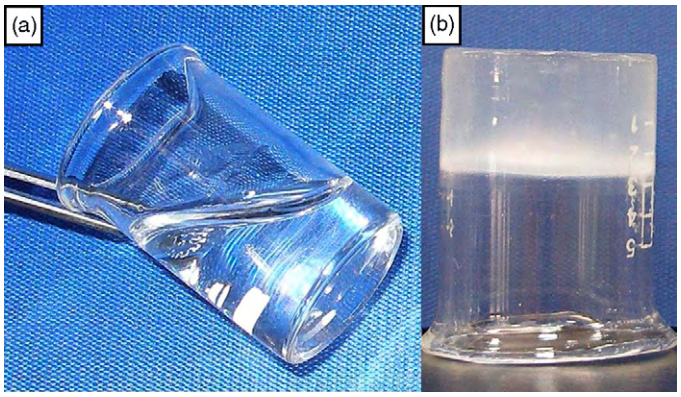

Fig. 1. (A) CS-HTCC/α,β-GP solution and (B) CS-HTCC/α,β-GP hydrogel at 37 °C.

2.7. Statistical analysis

All quantitative data were expressed as means ± standard deviation (SD). The *P*-values were calculated with a one-way ANOVA using SPSS13.0 statistical software (SPSS, USA) with $P < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Characterization of the CS-HTCC/GP thermosensitive hydrogel

The CS-HTCC/GP preparation remained in a liquid phase for the desired period of time when the temperature was below 25 °C (Fig. 1A) and turned into a gel state when the temperature was above 25 °C (Fig. 1B). This temperature-dependent phenomenon indicated that the prepared CS-HTCC/GP hydrogel was thermosensitive. The gelation temperature demonstrated a relationship with sol–gel transition time (Table 1). According to Table 1, the gelation time was 20 min at 25 °C, and the gel formed with less mechanical intensity. When the temperature was increased to 37 °C, the transition time increased to 3 min. The CS-HTCC/GP preparation exhibited adequate characteristics of response to external temperature changes between 35 and 37 °C. The sol–gel transition time was 3–5 min at normal body temperature (35–37 °C) and the gel formed exhibited good mechanical strength (Fig. 1B). The sol–gel transition time was shorter than both CS/α, β-GP (Chenite et al., 2000) and HTCC-PVA/α, β-GP thermosensitive hydrogels that have been described previously (Wu, Wei, Wang, Su, & Ma, 2007).

An SEM photograph (Fig. 2) demonstrated that the surface structure of the CS-HTCC/GP thermosensitive hydrogel became loose and many holes appeared in it, which made it favorable for water and small molecules to move freely in the network. This phenomenon can partly explain why the sol–gel transition time was significantly shortened.

Table 1
The relationship between different gelation temperature and gelation time.

CS+HTCC (%, w/v)	CS (%)	HTCC (%)	α,β-GP (%, v/v)	Gelation time (min)			
				25 °C	30 °C	35 °C	37 °C
2	83.33	16.67	9.09	10	8	3	3
2	83.33	16.67	8.33	20	11	10	3

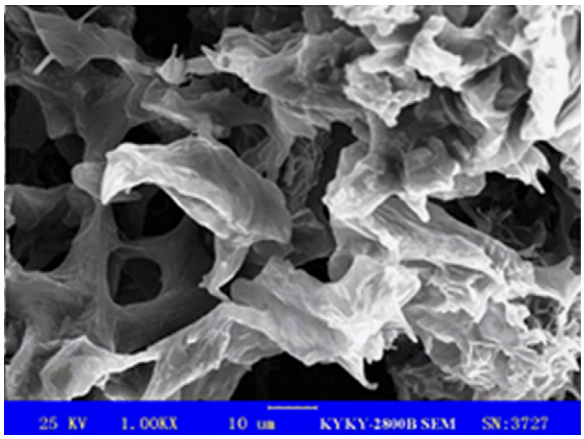

Fig. 2. SEM photomicrographs of the CS-HTCC/α,β-GP thermosensitive hydrogel.

3.2. MTT assay

The MTT assay is generally accepted as a routine method for establishing cytotoxicity of dental materials. The effects of the CS-HTCC/GP thermosensitive hydrogel on the viability of HPDLCs were determined using the MTT assay (Fig. 3). Incubation of the cells with different concentrations of extract for 1, 3, and 5 days resulted in significant differences compared with the controls (group 4). The concentration-dependent study (Fig. 3A) indicated that the CS-HTCC/GP thermosensitive hydrogel promoted HPDLC proliferation with various concentrations of CS-HTCC/GP extracts after 1, 3, and 5 days (groups 1–3). Compared with the control group (group 4), there was a significant increase in the number of HPDLCs at days 3 and 5 ($P<0.001$). Fig. 4B shows that HPDLC proliferation was significantly increased over 3 or 5 days compared to 1 day in the three hydrogel extract concentration groups. The MTT assay confirmed

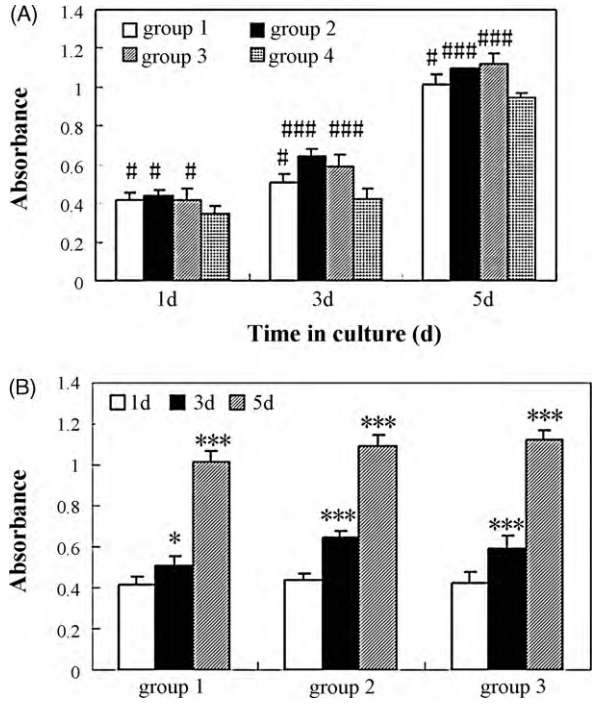

Fig. 3. Concentration-dependent (A) and time-dependent effects (B) of HPDLCs on the CS-HTCC/α,β-GP thermosensitive hydrogel extract was measured by MTT assay. The data were depicted as mean ± SD ($n=6$). $^{\#}P<0.05$, $^{\#\#\#}P<0.001$: significant difference compared to the controls (group 4) over the same culture time. $^{*}P<0.05$, $^{***}P<0.001$: significant differences at 1, 3 and 5 days in the same group.

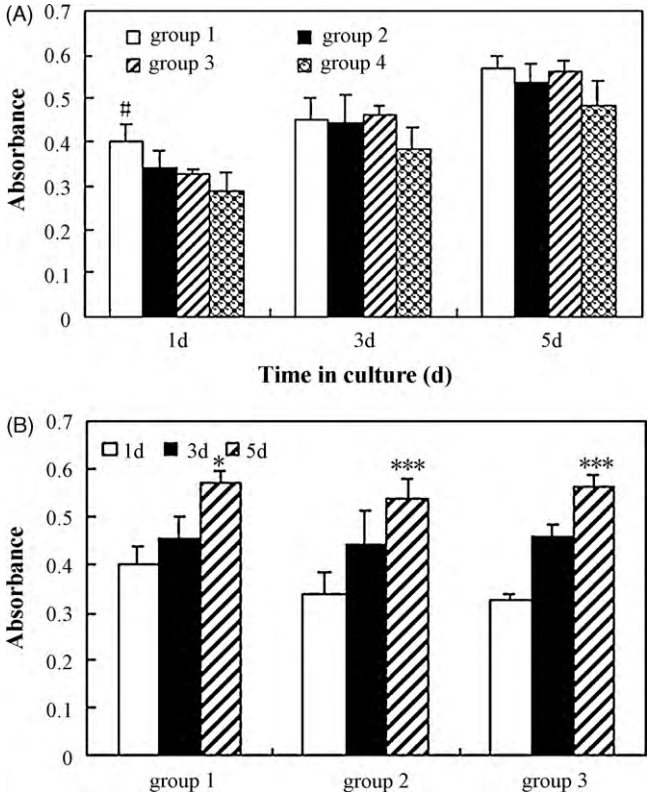

Fig. 4. Concentration-dependent (A) and time-dependent effects (B) of ALP activity in HPDLCs due to the CS-HTCC/α,β-GP thermosensitive hydrogel extract were measured with an ALP kit. The data depict the mean ± SD ($n=3$). $^{\#}P<0.05$: significant difference compared to the controls (group 4) over the same culture time.

that the CS/HTCC-GP thermosensitive hydrogel can promote the proliferation of HPDLCs. The cytotoxicity of these hydrogels was classified as grade I according to ISO 10993 (1997).

The PDL is a soft connective tissue located between the cementum and the alveolar bone that sustains and helps constrain teeth within the jaw. The PDL not only has an important role in supporting teeth, but also contributes to tooth nutrition, homoeostasis and repair of damaged tissue (Nojima et al., 1990; Somerman et al., 1990). The PDL contains heterogeneous cell populations that are able to differentiate into either cementum forming cells (cementoblasts) or bone-forming cells (osteoblasts) (Goseki et al., 1995; Molinaro, Leroux, Damas, & Adam, 2002; Nohutcu, McCauley, Koh, & Somerman, 1997). Many *in vitro* studies have shown that such cells produce type I collagen, ALP, osteonectin, osteopontin, and osteocalcin (Somerman et al., 1990; Takeshita et al., 1992). The presence of multiple cell types within the PDL has led to speculation that this tissue might contain progenitor cells that maintain tissue homoeostasis and are involved in the regeneration of periodontal tissue (Groeneveld, Everts, & Beertsen, 1995; Matsuda et al., 1993).

3.3. ALP assay

Fig. 4 outlines the results of ALP activity assay in HPDLCs affected by the CS-HTCC/GP thermosensitive hydrogel. The ALP activity was improved at days 1, 3 and 5 in all groups, with significant differences between groups 1 and 4 after 1 day ($P<0.05$). When comparing between days 1 and 5, a significant difference was found between groups 2 and 3 ($P<0.001$) and a significant difference was seen when compared to group 1 ($P<0.05$; Fig. 4B). These results showed that the CS-HTCC/GP thermosensitive hydrogel had obvious effects on ALP activity in HPDLCs.

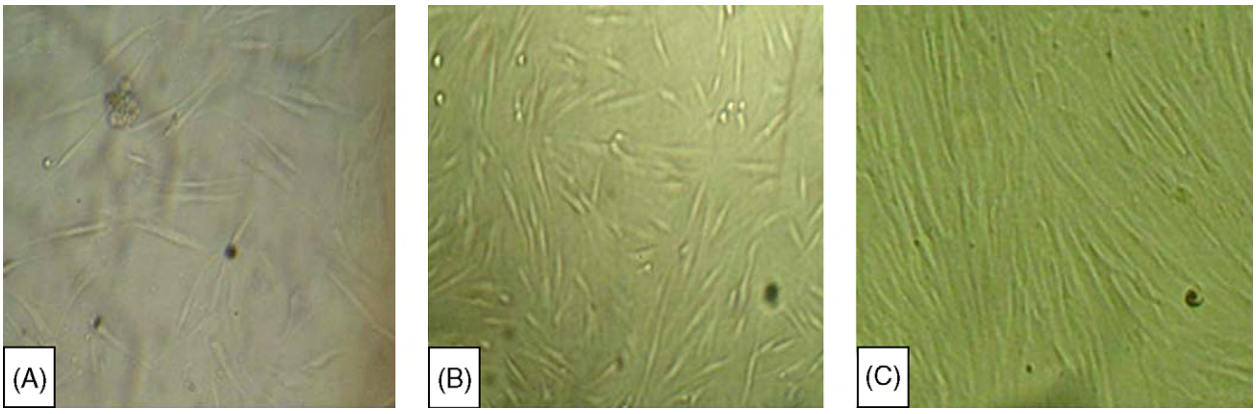

Fig. 5. Photomicrographs of HPDLCs in group 1 at 1, 3 and 5 days (100× magnification).

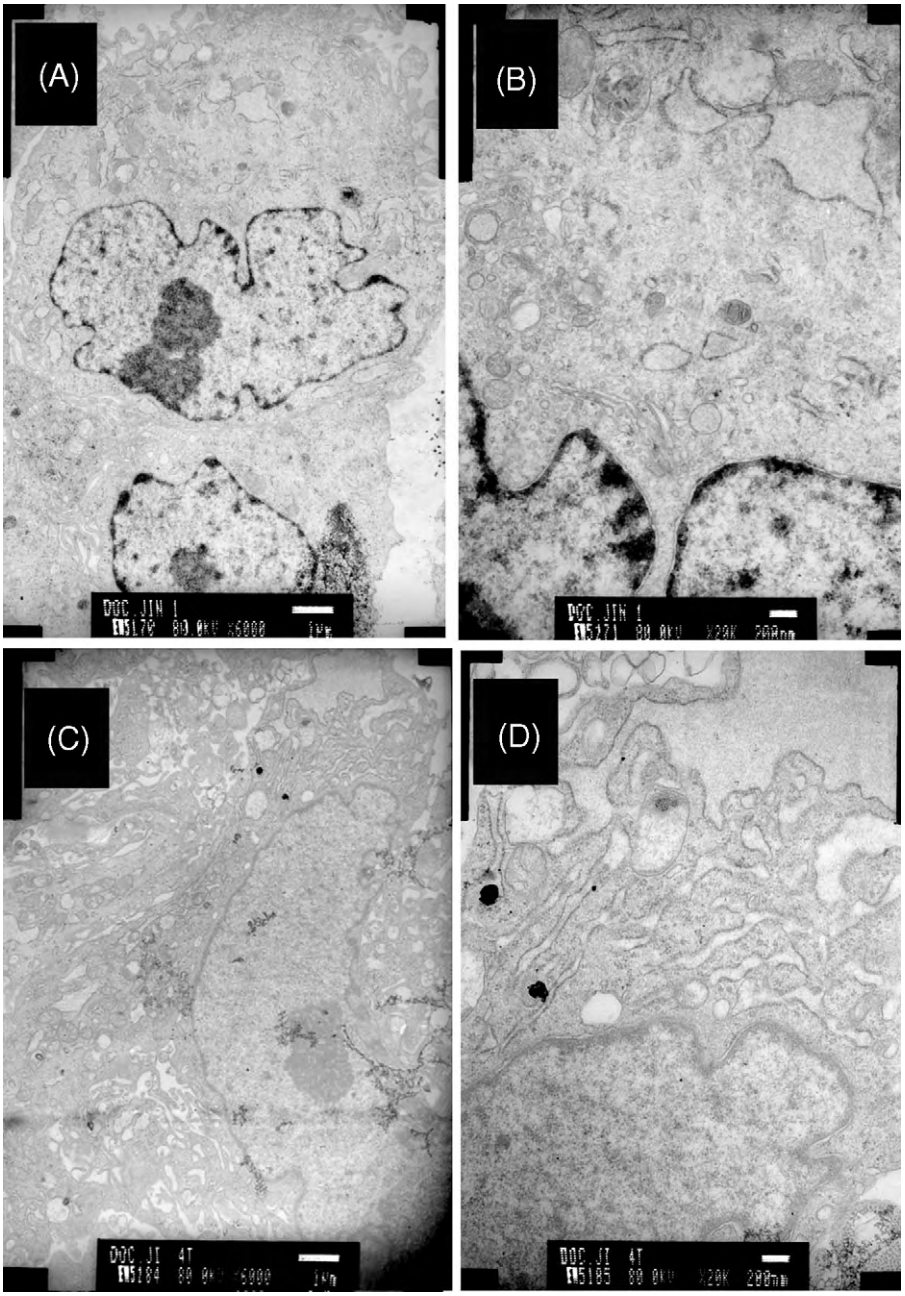

Fig. 6. TEM photomicrographs of HPDLCs in group 4 (A, 6000× magnification; B, 20,000× magnification) and group 1 (C, 6000× magnification; D, 20,000× magnification).

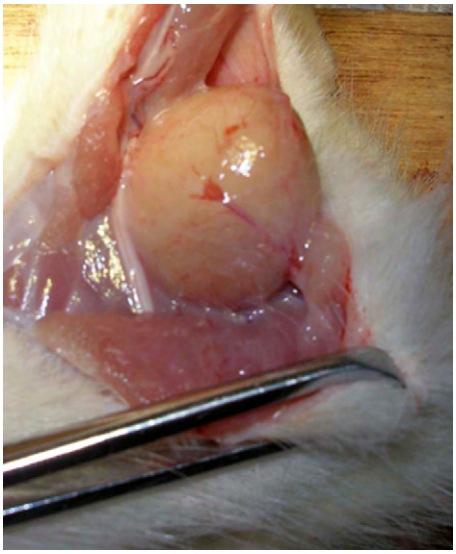

Fig. 7. The CS-HTCC/GP aqueous solution formed a round gel-like plug at the injection site.

3.4. The effects of CS-HTCC/GP thermosensitive hydrogel on HPDLC morphology

The morphological characteristics of the HPDLCs in group 1 at 1, 3 and 5 days are shown in Fig. 5A–C. The HPDLCs actively proliferated very quickly and had a good morphology. TEM revealed a difference in the ultrastructure of HPDLCs between groups 1 and 4 (Fig. 6). Cell membranes with good integrity and all kinds of cellular organelles could be observed in both groups. The ultrastructure of HPDLCs in group 1 (Fig. 6A and B) was quite similar to those in group 4 (Fig. 6C and D). No apoptotic signs such as condensed nuclear chromatin, extensive cytoplasmic vacuolization and blebbing were seen in the cells. This similarity in the ultrastructure of HPDLCs between groups 1 and 4 showed that even high concentrations of the CS-HTCC/GP thermosensitive hydrogel extract had no negative effect on the ultrastructure of HPDLCs. The results indicated that the CS-HTCC/GP thermosensitive hydrogel had good cytocompatibility.

3.5. In vivo histocompatibility of the CS-HTCC/GP thermosensitive hydrogel

The formation of the CS-HTCC/GP thermosensitive hydrogel *in vivo* was confirmed by injecting the CS-HTCC/GP aqueous solution (0.4 ml) intramuscularly into the rumps of Sprague–Dawley rats. Following injection, the CS-HTCC/GP aqueous solution formed a gel-like plug at the injection site as shown in Fig. 7, indicating that the CS-HTCC/GP aqueous solution gelled rapidly due to the increasing temperature of the tissue fluid surrounding the injection site. No other adverse reactions such as edema formation, hemorrhaging or discoloration were observed.

The inflammatory response at different times after injection could be characterized using HE staining. Fig. 8 represents the histological response of the implantation of the CS-HTCC/GP thermosensitive hydrogel. At 3 days post-injection, tissue swelling, degeneration and even necrosis appeared at the local injection site. The immediate infiltration by leukocytes, principally neutrophils, may well constitute the most important aspect of the acute inflammation reaction as demonstrated in Fig. 8A. In the following days, exudation and necrosis were observed. Granulation tissue, which is rich in fibroblasts and capillaries, appeared to encapsulate the gel and remaining necrotic tissue. The number of neutrophils was decreased while lymphocytes and macrophages were predominant at the injection site (Fig. 8B). The proliferation of fibroblasts was a manifestation of the repair process, and the number of neutrophils continued to decrease. Inflammation subsided, with the number of inflammatory cells, such as lymphocytes and macrophages, decreasing after 2 weeks, while the presence of collagen could also be demonstrated at these sites (Fig. 8C and D).

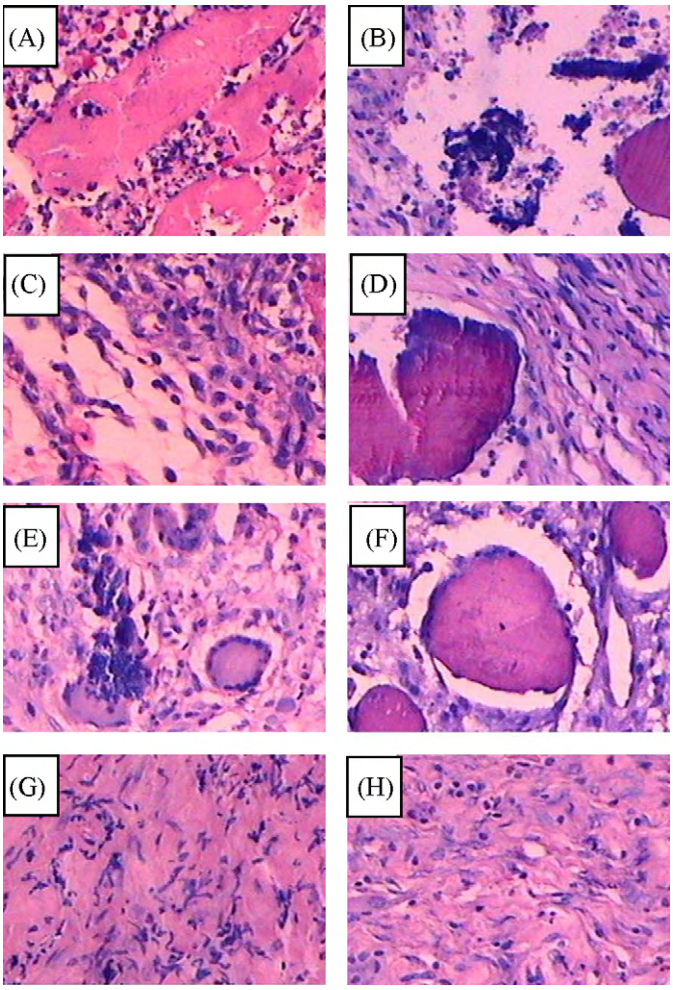

Fig. 8. Light microscopy investigation of the inflammatory reaction after intramuscular injection of the CS-HTCC/GP thermosensitive hydrogel in rats. (A) After 3 days, tissues were swollen with signs of necrosis (arrow) appearing at the local injection site. The leukocytes, principally neutrophils, (arrow) had infiltrated. (B) After 1 week, exudation and necrosis were observed. Granulation tissue encapsulated the gel (arrow) and necrotic tissue remained. Granulation tissue appeared and the number of neutrophils decreased along with inflammatory infiltration. (C and D) After 2 weeks, the number of lymphocytes and macrophages decreased while the amount of collagen increased. (E and F) After 4 weeks, giant cells formed by the fusion of macrophages engulfed the injected gel. Giant cells surrounded the injected gel and engulfed the gel (arrow indicates the giant cells and the gel). (G) After 6 weeks, the number of cells and vessels decreased continuously and the amount of collagen accumulated progressively. (H) After 9 weeks, a large amount of collagen had formed.

At 4 weeks, the number of fibroblasts and capillaries was decreased while the amount of collagen increased. As can be seen in Fig. 8E and F, giant cells formed by the fusion of macrophages and engulfed the injected gel. After 6 weeks, the number of cells and vessels decreased continuously and collagens progressively accumulated (Fig. 8G). At 9 weeks, cells and vessels were hardly seen and were replaced by a large amount of collagen (Fig. 8H).

In the inflammatory–reparative reaction, repair began soon after injection of the gel. All of the inflammation and repair pro-

cesses described above were physiologically normal reactions. Molinaro's study showed that the degree of inflammation was related to the injection site because of blood flow, tight geometry of injection space and other factors (Molinaro et al., 2002). In the present study, the hydrogel had degraded substantially *in vivo* and the affected region was covered with connective tissue as time progressed. These results indicate that the CS-HTCC/GP thermosensitive hydrogel was fully biocompatible *in vivo*.

3.6. Implantation of CS-HTCC/GP thermosensitive hydrogel in dog periodontal defect models

No animals died during the experimental procedure. The tissue specimens were stained by Mallory's trichrome staining methods and the histological findings were shown in Fig. 9. The regenerative tissues completely filled the fraction areas. There was a significant amount of new bone and cementum, along with PDLs and coronal

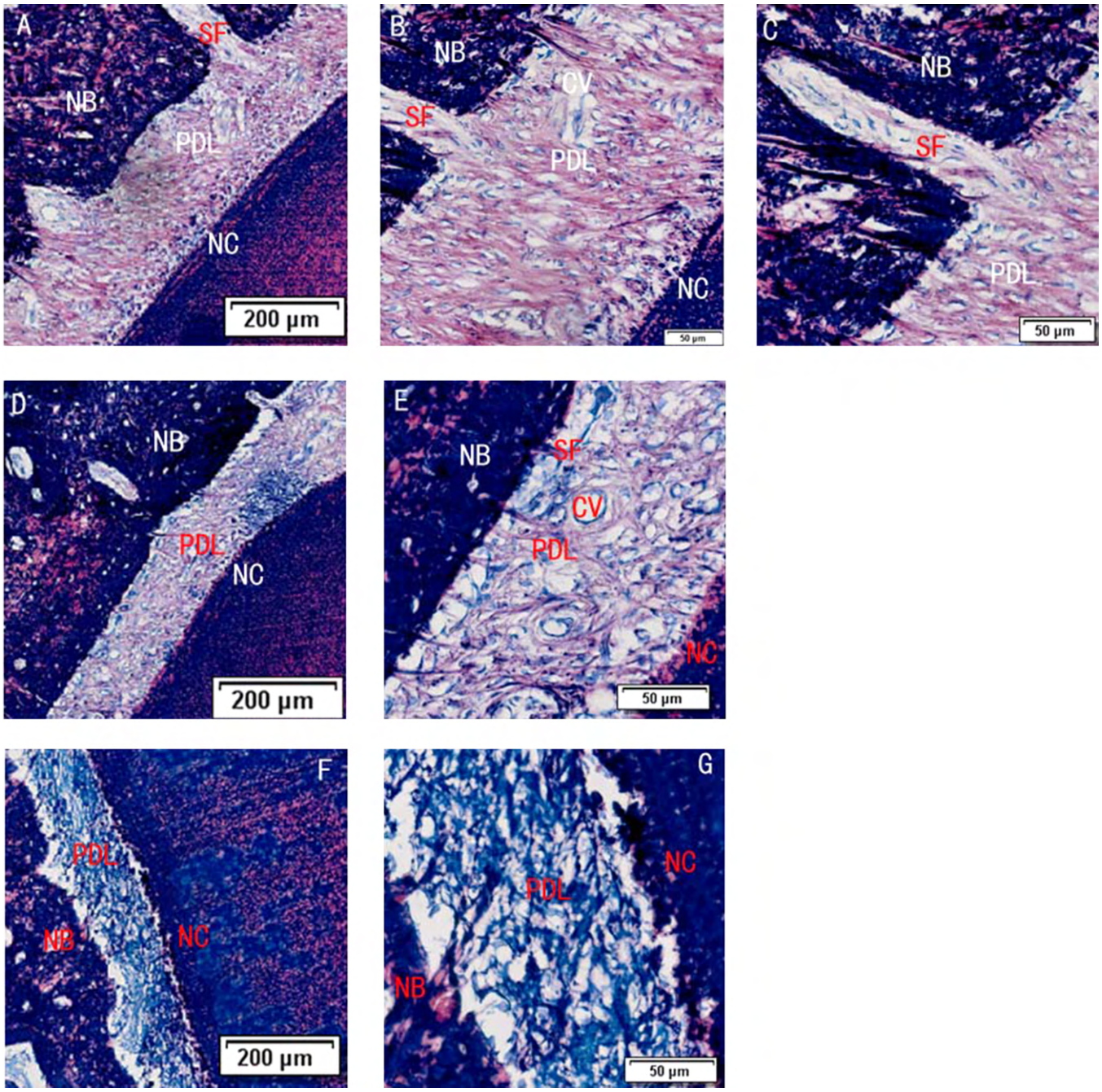

Fig. 9. Histological examination of periodontal tissue regeneration (Mallory's trichrome staining methods). NB, new bone; NC, new cementum; PDL, new periodontal ligaments; SF, Sharpey's fibers; CV, capillary vessel in different groups over 8 weeks post-surgery (A, D and F: 40× magnification; C, E and G: 100× magnification; and B: 80× magnification). In the CS-HTCC/GP-bFGF group (A, B and C), a significant amount of NB and NC, along with PDL and coronal growth alveolar bone, was observed. The regulated and sturdy SFs were inserted into the alveolar, and all periodontal tissues including CVs were reconstructed with an adequate width of PDLs, which separated the NB from the cementum (B). In the CS-HTCC/GP group, all the periodontal tissues did not exhibit full regeneration and few periodontal tissues such as regular PDL, cementum and new bone could be observed (D and E) compared with the CS-HTCC/GP-bFGF group. SF and bone reconstruction could also be seen (D). (E) is the magnified view of the frame from (C). Less PDL or hard tissue regeneration was observed in the negative control group (F and G) and almost no cementum regeneration was observed in the defect area (F).

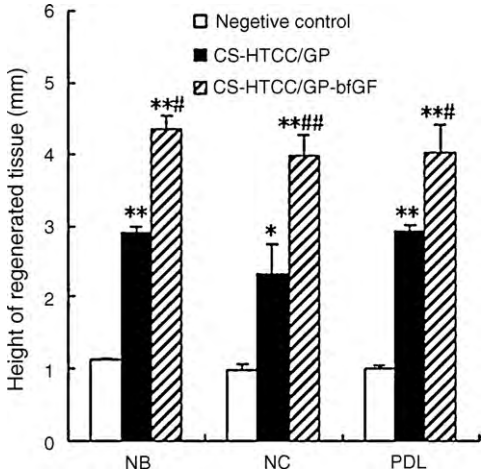

Fig. 10. The height measurement of regeneration tissue in the three groups over 8 weeks post-surgery (NB, new bone; NC, new cementum; PDL, new periodontal ligaments). $^{*}P<0.05$, $^{**}P<0.01$: significant *vs.* the values of the CS-HTCC/GP thermosensitive hydrogel. $^{\#}P<0.05$: significant *vs.* the values of negative control group.

growth alveolar bone in the CS-HTCC/GP-bFGF group (Fig. 9A–C). The NC was on the surface of the denuded root. The regulated and sturdy Sharpey's Fibers (SF) were inserted into the alveolar, and all periodontal tissues including the capillary vessel (CV) were reconstructed with adequate width of PDLs, which separated the NB from the cementum (Fig. 9B). Few periodontal tissues such as regular PDL, cementum and new bone could be observed (Fig. 9D and E) in the CS-HTCC/GP group compared with the CS-HTCC/GP-bFGF group. In the negative control group, there was less NB, less NC and less PDL regeneration (Fig. 9F and G).

The height measurement of regeneration tissue in the three groups can be seen in Fig. 10. The values in the CS-HTCC/GP-bFGF and CS-HTCC/GP groups were significantly higher than those in the negative control group. The CS-HTCC/GP thermosensitive hydrogel group achieved the highest value. Statistical differences were also found between the CS-HTCC/GP-bFGF and CS-HTCC/GP groups.

4. Conclusions

In this study, a CS-HTCC/GP thermosensitive hydrogel was developed. The hydrogel remained in aqueous solution below 25 °C and turned into a gel when the temperature was greater than the normal human body temperature. The CS-HTCC/GP thermosensitive hydrogel significantly enhanced proliferation and ALP activity in HPDLCs. HPDLCs maintained good morphology in the 50% (v/v) CS-HTCC/GP thermosensitive hydrogel extract. When the hydrogel solution was injected into the rumps of rats, it transformed into a gel and inhibited biocompatibility. The CS-HTCC/GP thermosensitive hydrogel was shown to have an effect upon periodontal tissue regeneration as well as being useful as a tissue-engineering scaffold. When conjugated with bFGF, its tissue regeneration ability was strengthened. In summary, the CS-HTCC/GP thermosensitive hydrogel was proven to be highly suitable as an injectable drug delivery vehicle, as well as a tissue-engineering scaffold in a single device for periodontal disease therapy.

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References

Barreiro-Iglesias, R., Coronilla, R., Concheiro, A., & Alvarez-Lorenzo, C. (2005). Preparation of chitosan beads by simultaneous cross-linking/insolubilisation in basic pH. Rheological optimisation and drug loading/release behaviour. *European Journal of Pharmaceutical Sciences*, *24*(1), 77–84.

Camps, J., & About, I. (2003). Cytotoxicity testing of endodontic sealers: a new method. *Journal of Endodontics*, *29*(9), 583–586.

Chen, F. M., Zhao, Y. M., Wu, H., Deng, Z. H., Wang, Q. T., Zhou, W., et al. (2006). Enhancement of periodontal tissue regeneration by locally controlled delivery of insulin-like growth factor-I from dextran-co-gelatin microspheres. *Journal of Controlled Release*, *114*(2), 209–222.

Chenite, A., Chaput, C., Wang, D., Combes, C., Buschmann, M. D., Hoemann, C. D., et al. (2000). Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*, *21*(21), 2155–2161.

Cho, J. H., Kim, S. H., Park, K. D., Jung, M. C., Yang, W. I., Han, S. W., et al. (2004). Chondrogenic differentiation of human mesenchymal stem cells using a thermosensitive poly(*N*-isopropylacrylamide) and water-soluble chitosan copolymer. *Biomaterials*, *25*(26), 5743–5751.

Chung, Y. M., Simmons, K. L., Gutowska, A., & Jeong, B. (2002). Sol–gel transition temperature of PLGA-g-PEG aqueous solutions. *Biomacromolecules*, *3*(3), 511–516.

GB/T16886.1. (2001). *Biological evaluation of medical devices—Part 1: Evaluation and testing*.

Goseki, M., Oida, S., Takeda, K., Ogata, Y., Iimura, T., Maruoka, Y., et al. (1995). Identification of bone-type alkaline phosphatase mRNA from human periodontal ligament cells. *Journal of Dental Research*, *74*(1), 319–322.

Groeneveld, M. C., Everts, V., & Beertsen, W. (1995). Alkaline phosphatase activity in the periodontal ligament and gingiva of the rat molar: its relation to cementum formation. *Journal of Dental Research*, *74*(7), 1374–1381.

ISO10993-1. (1997). *Biological evaluation of medical devices—Part 1: Evaluation and testing*.

Ji, Q. X., Chen, X. G., Zhao, Q. S., Liu, C. S., Cheng, X. J., & Wang, L. C. (2009). Injectable thermosensitive hydrogel based on chitosan and quaternized chitosan and the biomedical properties. *Journal of Materials Science. Materials in Medicine*, *20*(8), 1603–1610.

Lagarce, F., Renaud, P., Faisant, N., Nicolas, G., Cailleux, A., Richard, J., et al. (2005). Baclofen-loaded microspheres: preparation and efficacy testing in a new rabbit model. *European Journal of Pharmaceutical Sciences*, *59*(3), 449–459.

Langer, R., & Peppas, N. (2003). Advances in biomaterials, drug delivery and bionanotechnology. *AIChE Journal*, *12*, 2990–3006.

Leeuwenburgh, S. C., Jansen, J. A., Malda, J., Dhert, W. A., Rouwkema, J., van Blitterswijk, C. A., et al. (2008). Trends in biomaterials research: An analysis of the scientific programme of the World Biomaterials Congress 2008. *Biomaterials*, *29*(21), 3047–3052.

Matsuda, N., Kumar, N. M., Ramakrishnan, P. R., Lin, W. L., Genco, R. J., & Cho, M. I. (1993). Evidence for up-regulation of epidermal growth-factor receptors on rat periodontal ligament fibroblastic cells associated with stabilization of phenotype *in vitro*. *Archives of Oral Biology*, *38*(7), 559–569.

Molinaro, G., Leroux, J. C., Damas, J., & Adam, A. (2002). Biocompatibility of thermosensitive chitosan-based hydrogels: an *in vivo* experimental approach to injectable biomaterials. *Biomaterials*, *23*(13), 2717–2722.

Nohutcu, R. M., McCauley, L. K., Koh, A. J., & Somerman, M. J. (1997). Expression of extracellular matrix proteins in human periodontal ligament cells during mineralization *in vitro*. *Journal of Periodontology*, *68*(4), 320–327.

Nojima, N., Kobayashi, M., Shionome, M., Takahashi, N., Suda, T., & Hasegawa, K. (1990). Fibroblastic cells derived from bovine periodontal ligaments have the phenotypes of osteoblasts. *Journal of Periodontal Research*, *25*(3), 179–185.

Quirynen, M., Teughels, W., & van Steenberghe, D. (2003). Microbial shifts after subgingival debridement and formation of bacterial resistance when combined with local or systemic antimicrobials. *Oral Diseases*, *9*(Suppl. 1), 30–37.

Roberts, M. C. (2002). Antibiotic toxicity, interactions and resistance development. *Periodontology 2000*, *28*, 280–297.

Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J., et al. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet*, *364*(9429), 149–155.

Somerman, M. J., Young, M. F., Foster, R. A., Moehring, J. M., Imm, G., & Sauk, J. J. (1990). Characteristics of human periodontal ligament cells *in vitro*. *Archives of Oral Biology*, *35*(3), 241–247.

Southard, G. L., & Godowski, K. C. (1998). Subgingival controlled release of antimicrobial agents in the treatment of periodontal disease. *International Journal of Antimicrobial Agents*, *9*(4), 239–253.

Takeshita, A., Niu, Z. G., Hanazawa, S., Takara, I., Higuchi, H., Katayama, I., et al. (1992). Effect of interleukin-1 beta on gene expressions and functions of fibroblastic cells derived from human periodontal ligament. *Journal of Periodontal Research*, *27*(4 Pt 1), 250–255.

Tatakis, D., & Kumar, P. (2005). Etiology and pathogenesis of periodontal diseases. *Dental Clinics of North America*, *49*, 491–516.

van Winkelhoff, A. J., Herrera Gonzales, D., Winkel, E. G., Dellemijn-Kippuw, N., Vandenbroucke-Grauls, C. M., & Sanz, M. (2000). Antimicrobial resistance in the subgingival microflora in patients with adult periodontitis. A comparison between The Netherlands and Spain. *Journal of Clinical Periodontology*, *27*(2), 79–86.

Williams, D. F. (2008). On the mechanisms of biocompatibility. *Biomaterials*, *29*(20), 2941–2953.

Wu, J., Wei, W., Wang, L. Y., Su, Z. G., & Ma, G. H. (2007). A thermosensitive hydrogel based on quaternized chitosan and poly(ethylene glycol) for nasal drug delivery system. *Biomaterials*, *28*(13), 2220–2232.