Carbohydrate Polymers 113 (2014) 240–248

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

A comparison of physicochemical properties of sterilized chitosan hydrogel and its applicability in a canine model of periodontal regeneration

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 23 June 2014
Accepted 2 July 2014
Available online 16 July 2014

Keywords:
Chitosan
Periodontal tissue engineering
Thermosensitive hydrogel
Scaffold

ABSTRACT

Chitosan has previously been exploited as a scaffold in tissue engineering processes. To avoid infection, chitosan must be sterilized prior to contact with bodily fluids or blood. Previous research has shown that autoclaved chitosan solution lead to decreased molecular weight, dynamic viscosity, and rate of gelling. We prepared a thermosensitive chitosan hydrogel using autoclaved chitosan powder (121 °C, 10 min) and β-glycerophosphate (chitosan-PA/GP) and compared the physicochemical properties and biocompatibility *in vitro* with autoclaved chitosan solution/GP hydrogel. The chitosan-PA/GP hydrogel had a shortened gelation time, higher viscosity, increased water absorption, appropriate degradation time, porous structure, and no obvious cytotoxicity on human periodontal ligament cells. Scanning electron microscopy demonstrated that the cells exhibited a normal morphology. The chitosan-PA/GP hydrogel promoted periodontal tissue regeneration in dog class III furcation defects. The chitosan-PA/GP thermosensitive hydrogel displayed suitable physicochemical properties and biocompatibilities and represents a promising candidate as an injectable tissue engineering scaffold.

1. Introduction

In periodontal tissue engineering, the ultimate goal is to create a space in which functional cells can grow (Bartold, McCulloch, Narayanan, & Pitaru, 2000). To this end, different types of scaffolds, such as hydrogels, have been applied during periodontal treatment. Chitosan is widely used in medical treatments and for wound healing processes, including periodontal tissue engineering, in accordance with its non-toxic, biodegradable, biocompatible, adhesive, and antibacterial properties (Mattioli Belmonte et al., 1998; Muzzarelli., 1993; Muzzarelli, 2009, 2010). When combined with β-glycerophosphate (GP), chitosan/GP can be maintained stably in solution at room temperature for extended periods of time. At physiological temperatures (37 °C), the chitosan/GP complex turns into a hydrogel, which may improve its clinical applicability and operative convenience. This complex can also be loaded with drugs (Barreiro-Iglesias, Coronilla, Concheiro, & Alvarez-Lorenzo, 2005; Chen, Tian, & Du, 2004) that release slowly over time and may even function as a scaffold for cell growth (Ji, Khademhosseini, & Dehghani, 2011; Niranjan et al., 2013).

Sterilization of the chitosan/GP hydrogel is required prior to implantation in the body or contact with bodily fluids. Previous research has demonstrated that the physical, chemical, mechanical, and biological properties of biomaterials can be negatively affected by sterilization (Jarry et al., 2001; Jarry, Leroux, Haeck, & Chaput, 2002; Lim, Khor, & Ling, 1999). Autoclaving, dry heat, ultraviolet radiation, gamma radiation, ethylene oxidation, and immersion in alcohol aqueous solutions are all methods previously exploited to sterilize chitosan. Ethylene oxide and gamma radiation have adequate effectiveness and are compatible with a wide array of materials. However, ethylene oxide is highly flammable, produces toxic residues, and has environmental exposure risks. Gamma radiation negatively affected polymer properties and performance due to material-intrinsic sensitivities or susceptibilities. Ultraviolet radiation was previously shown to cause accelerated degradation of chitosan (Yue, He, Yao, & Wei, 2009). Because ethanol is not a true

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http://dx.doi.org/10.1016/j.carbpol.2014.07.018
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sterilizing agent, it does not kill the endospores of many bacteria (Holy, Cheng, Davies, & Shoichet, 2001).

Steam sterilization is a simple and effective process that does not produce toxic residues. It is currently considered to be the most practical means of sterilizing medical devices and fluids. After sterilization by autoclave or gamma radiation, the gelation time of chitosan solution was increased significantly (Peng et al., 2012; Jarry et al., 2001). Most biomedical polymers, however, are hydrolyzed, degraded and soften under the high temperature and pressure conditions of steam sterilization. Previous studies have reported the effect of different sterilization methods on the chitosan membranes and injectable chitosan hydrogel (Marreco, da Luz Moreira, Genari, & Moraes, 2004; Rao & Sharma, 1995). After sterilization, however, the chitosan membranes or chitosan/GP hydrogel have proven difficult to supplement with cells or growth factors due to thermosensitive behavior. Steam autoclaving is often used to sterilize the chitosan solution prior to the addition of GP (Jarry et al., 2001). Although the chitosan molecular weight (MW) is reduced following the autoclaving process, the hydrogels that have been prepared using autoclaved chitosan did have the desired thermosensitivity for biological-relevant applications and this autoclaving method was deemed suitable for sterilization (Jarry et al., 2002). In addition, Yang *et al.* (Yang, Zhao, Liu, Ding, & Gu, 2007) demonstrated that the steam sterilization process caused no change in the chemical structure of chitosan (powder form). For this study, we selected the steam sterilization method to treat chitosan powder, prior to solution preparation, and already-mixed chitosan solution and evaluated its effect on the physicochemical properties of the chitosan.

The proportion of chitosan to GP in the hydrogel can vary and volume:volume ratios of 9:1 (Chenite et al., 2000) and 7:1 (Wang, Yang, Duan, Li, & Zhang, 2006) have both been reported in investigative research studies. It is still necessary to establish what the optimal ratio is for cell proliferation and adhesion within the chitosan/GP gel. Ji et al. (Ji et al., 2009a, 2010) previously published a quaternized chitosan (CS-HTCC) containing α,β-GP (CS-HTCC/GP) gel and supplemented with bFGF in a canine model of periodontal regeneration. The final viscosity of their CS-HTCC/GP hydrogel, however, was low, which may limit its application in tissue engineering. The observed periodontal repair may be due in part to the co-application of bFGF. On the other hand, our previous report showed that (Peng et al., 2012) gelation time for chitosan/GP was increased significantly after sterilization by autoclave or gamma radiation (Jarry et al., 2001). The present study was designed to investigate systematically the influence of steam sterilization methods on the physicochemical properties, cytocompatibility, and growth of human periodontal ligament cells (HPDLCs) in chitosan/GP without the addition of growth factors. Additionally, sterilized chitosan/GP thermosensitive hydrogels were implanted in a canine model of periodontal defects to evaluate regenerative potential.

2. Materials and methods

2.1. Preparation of the chitosan/GP solution

The solution of autoclaved chitosan powder (chitosan-PA) was prepared by adding 200 mg autoclaved (dry cycle; 121 °C for 10 min) chitosan (median MW, 75–85% degree of deacetylation (DD); Catalogue No. 448877; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) to 9 mL hydrochloric acid (0.1 mol/L), stirring until completely dissolved. The autoclaved chitosan solution (chitosan-SA) was prepared by adding 200 mg chitosan to 9 mL hydrochloric acid (0.1 mol/L) while stirring; the samples of chitosan solution were then autoclaved at 121 °C for 10 min (Jarry et al., 2001). The primary difference between these two groups is the process sequence of the chitosan sterilization step, which occurred either before (chitosan powder) or after (chitosan solution) the gelation step. Both solutions were processed in a similar manner thereafter. β-glycerophosphate (GP) (E. Merck, Darmstadt, Germany) (560 mg) was dissolved in 1 mL distilled water and sterilized by filtration. Before use, both the chitosan and GP solutions were chilled in an ice bath for 15 min to avoid gelation. The 1 mL GP solution was then added dropwise to the 9 mL chitosan-SA with continuous stirring to form a clear homogeneous chitosan-SA/GP (9:1, volume:volume) solution. The chitosan-PA/GP (9:1) and chitosan-PA/GP (7:1) solutions were prepared as described above but with the autoclaved chitosan powder. Preparation of the chitosan-PA/GP (9:1), of the chitosan-PA/GP (7:1), and of the chitosan-SA/GP (9:1) was performed on a clean bench and all prepared solutions were sealed and stored at 4 °C until use.

2.2. Determination of chitosan/GP thermosensitive hydrogel properties

The chitosan/GP hydrogel was placed in a 37 °C water bath. Using the test tube inversion method (Chung, Simmons, Gutowska, & Jeong, 2002), we assayed the thermosensitivity of chitosan-PA/GP and chitosan-SA/GP hydrogels. The sol-to-gel behavior of chitosan/GP thermosensitive hydrogels was further studied by measuring the sample solution viscosity at predetermined time intervals using a viscometer at 37 °C.

The obtained chitosan hydrogels were frozen at −80 °C for 24 h and then lyophilized in a freeze dryer (LGJ-10; Xi'an Zhongnuo Co., Ltd., China) for 24 h at −40 °C (Yang et al., 2010). The surfaces of chitosan/GP hydrogels were coated with a gold–palladium layer and observed using scanning electron microscopy (SEM) (S-4800, HITACHI, Japan). Mean pore diameters were analyzed by digital SEM photos of sectioned samples.

2.3. Swelling studies

Chitosan/GP hydrogels were weighed and fully rehydrated in a sealed container with phosphate buffered saline (PBS; pH 7.4) as previously described (Chen et al., 2004) for 24 h at 37 °C to study the swelling characteristics. Samples were taken out of PBS and the surface was dried with filter paper and weighed again. The swelling ratio was determined gravimetrically using following equation:

$$D_s\,(\%) = \frac{W_s - W_0}{W_0} \times 100\%,$$

where W_0 is the dry weight and W_s is the saturated weight.

2.4. Biodegradability

Chitosan/GP hydrogel was prepared at a 2 mm thickness and 1 cm diameter and incubated in 2 mL of DMEM medium with 0.1% (w/v) sodium azide (Wolsen Biotechnology Co. Ltd., Shanxi, China) containing 500 μg/mL chicken egg white lysozyme (Wolsen Biotechnology Co. Ltd., Shanxi, China). As a control, hydrogel was also treated with same medium but without lysozyme. The samples were then put into a 37 °C incubator for 21 days and the medium was replenished weekly. Dry weights of the samples were measured on the 1st, 4th, 7th, 14th, and 21st days of incubation. Degradation was determined by percentage of weight loss (W_L) using following equation:

$$W_L\,(\%) = \frac{W_L - W_0}{W_0} \times 100\%,$$

where W_0 was the initial weight and W_L was the weight after degradation.

2.5. Cell culture

2.5.1. HPDLC isolation and culture conditions

HPDLCs were acquired from extracted premolars of orthodontic patients (12–17 years old) without gingivitis or periodontitis. Informed consent was obtained from all patients under a protocol approved by the Ethical Committee of Stomatological Hospital of FMMU, PLA, China (IRB-REV-2013006). HPDLCs were obtained and cultured as previously described (Pang et al., 2005), using passage 3–5 cells.

2.5.2. MTT assay

According to ISO10993-5 (ISO10993-1, 1997), the ratio between the sample surface and the volume of the medium was 0.5 cm^2/mL. The cytotoxicity of the hydrogel was evaluated by an extraction test (Camps & About, 2003). In brief, cells were cultured in a 96-well plate at a density of 1×10^3 cells/well in DMEM and 10% fetal bovine serum (FBS) for 24 h. The cells were then divided into four groups and separately treated with chitosan-SA/GP (9:1) extract, chitosan-PA/GP (9:1) hydrogel extracts, chitosan-PA/GP (7:1) hydrogel extracts, or a negative control of DMEM alone. The medium was replaced every 2 days. At days 1, 3, 5, and 7 of incubation, the proliferative capacity of cells in each group was examined by the MTT method (Ji et al., 2009b). The optical density (OD) of each well was measured at 450 nm using a PowerWave 340 automated microplate reader (BIO-TEK, Winooski, VT, USA). The assay results for different groups were recorded and compared by statistical analysis.

2.5.3. Cell adhesion and viability

Sterilized hydrogel was added to 24-well culture plates and immersed in DMEM and 10% FBS overnight. A 1×10^5 cell suspension in 100 μL was seeded onto the hydrogel. After 3 h, another 900 μL culture medium was added to each well, and the culture was maintained at 37 °C in a humidified 5% CO_2 atmosphere. After 1 and 7 days, the HPDLCs were fixed in 2.5% glutaraldehyde for 12 h for staining and SEM observation. Each well was washed with PBS three times and 0.1% Triton X-100 was then added to each well. Each well was then washed with PBS for an additional three times prior to addition of 4′,6-diamidino-2-phenylindole (DAPI; Wolsen Biotechnology Co. Ltd., Shanxi, China). Immunofluorescence images were visualized under an inverted microscope (DMI6000B; Leica, Wetzlar, Germany). The remaining cell–hydrogel complexes were sputter coated with gold–palladium and observed by SEM (S-4800; Hitachi, Tokyo, Japan)

2.6. Implantation of chitosan/GP thermosensitive hydrogel in class III furcation defects in a canine model

Six healthy male mixed breed dogs (15 months old; 15.0 ± 2.0 kg) were used for this experiment. The Institutional Committee on Animal Research of the Stomatological Hospital of FMMU, PLA, China approved the protocol for this research. Class III furcation defects (Fig. 1) were generated as previously described (Roriz, Souza, Taba, Palioto, & Grisi, 2006). The bilateral mandibular third and fourth premolars in each of the six dogs were selected for study and the defects were randomly assigned to the following three treatment groups: (1) chitosan-SA/GP (9:1) hydrogel ($n = 8$), (2) chitosan-PA/GP (9:1) hydrogel ($n = 8$), and (3) negative control ($n = 8$). The sterilized chitosan/GP thermosensitive hydrogels were implanted into the defects. Twelve weeks after treatment, the six dogs were anesthetized and sacrificed so that the oral tissues could be fixed via perfusion with 10% buffered formalin. The samples were decalcified, dehydrated, embedded in paraffin, and stained via the Van Gieson's staining method (Araujo, Berglundh, & Lindhe, 1996). Histological and histometric analyses were performed using a Leica fluorescence microscope (DM6000B).

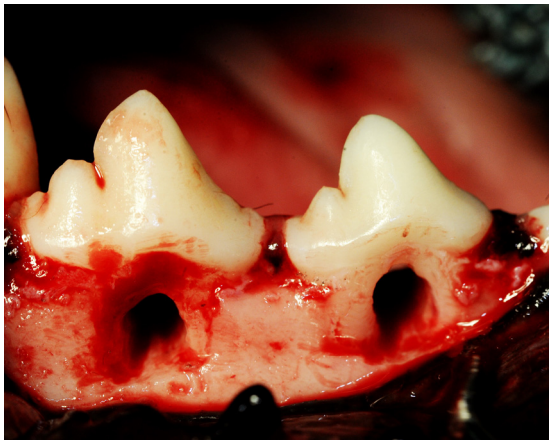

Fig. 1. Surgical procedure. Class III furcation defects were prepared (5 × 4 mm).

2.7. Statistical analysis

All data are shown as the mean with standard deviation (SD) and results were analyzed with a one-way ANOVA via SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). A P value of less than 0.05 was set as the threshold for statistical significance.

3. Results and discussion

3.1. Chitosan/GP thermosensitive hydrogel properties

3.1.1. Gelling properties and SEM observations

At 25 °C, the chitosan/GP solution remained a liquid. When the temperature was increased to 37 °C, the chitosan/GP that existed in a flowing liquid sol phase was transformed into a non-flowing hydrogel (Fig. 2A–C). All three samples had a porous structure, as observed via SEM (Fig. 2D–F). The average pore size of the three hydrogels were 186.2 ± 38.2 μm in the chitosan-SA/GP (9:1) group, 174.5 ± 35.6 μm in the chitosan-PA/GP (9:1), and 170.9 ± 37.8 μm in the chitosan-SA/GP (7:1) group. When assessed through SEM, the morphology of the three hydrogels was not affected by sterilization treatments. There was no statistically significant difference among the three groups.

The pH values (Fig. 3A) of the chitosan-PA/GP (9:1 and 7:1) were within a physiologically neutral range (from 6.8 to 7.2), with the chitosan-SA/GP (9:1) group having a lower pH range (from 6.5 to 6.9). We observed a significant difference in gelation time (Fig. 3A). While the chitosan-PA/GP (9:1 and 7:1) groups both formed a hydrogel in about 5–6 min, the chitosan-SA/GP (9:1) group took upwards of 30 min, and the difference in time was statistically significant ($P < 0.05$). The autoclaved chitosan powder combined with GP transitioned more easily into a hydrogel at body temperature. Ji et al. prepared CS-HTCC/GP gel by adding HTCC and α,β-GP, which both greatly affected the gelation time. The higher concentration of HTCC and α,β-GP decreased the gelation. Although our sol–gel transition time was approximately 5–6 min, which was a little longer than Ji et al., it did not affect the capacity of gelation or the clinical applicability.

Several physicochemical parameters may impact the chitosan/GP thermosensitive complex, such as the DD, MW, chitosan structure, GP concentration, pH value, and temperature. Jarry et al. (Jarry et al., 2001) reported that steam-sterilized chitosan solutions have a reduced MW, which decreases the gelling rate of the chitosan/GP hydrogel. In addition, Yang et al. (Yang et al., 2007)

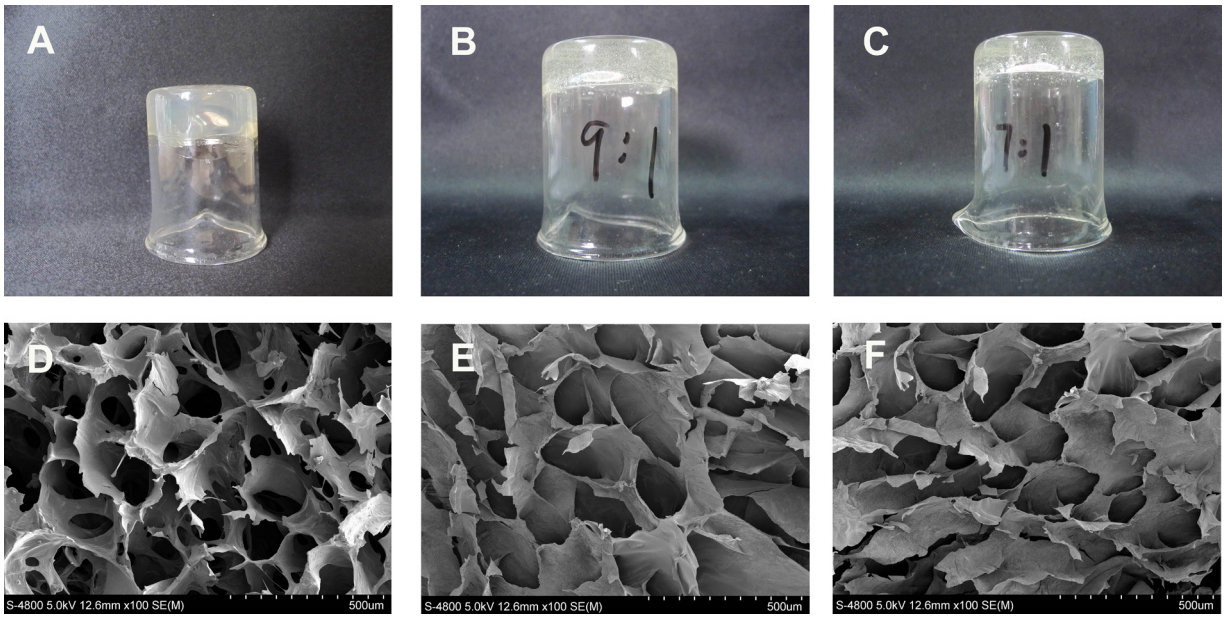

Fig. 2. Chitosan/GP hydrogel images. (A) The chitosan-SA/GP (9:1) group. (B) The chitosan-PA/GP (9:1) group. (C) The chitosan-PA/GP (7:1) group. SEM micrographs of chitosan gelation. (D) The chitosan-SA/GP (9:1) group (×100). (E) The chitosan-PA/GP (9:1) group (×100). (F) The chitosan-PA/GP (7:1) group (×100).

reported that no obvious change occurred in the DD and MW of chitosan during the steam sterilization step, and Lim et al. (Lim et al., 1999) reported that the heating conditions promote the cross-linking of the polymer in chitosan, ultimately decreasing its water solubility. Hence, chitosan-SA/GP (9:1) hydrogels, which were prepared by autoclaving-sterilizing the pre-made chitosan solution, may have a longer gelation time than the chitosan-PA/GP (9:1) or chitosan-PA/GP (7:1), which were made by autoclaving-sterilizing the chitosan prior to mixing it in solution. The results of the current study showed lower gelation times than previously reported (Chenite et al., 2000; Ruel-Gariepy, Chenite, Chaput, Guirguis, & Leroux, 2000). Steam sterilization of chitosan may have different effects depending on whether sterilization was performed during its dry state or after it was in solution. Our initial sterilization experiments were conducted on dry state chitosan. We found that the autoclave sterilization process caused chitosan to assume a darker color, similar to what has been previously published (Larena, Cáceres, Vicario, & Fuentes, 2004).

3.1.2. Viscosity measurements

Fig. 3B illustrates the variation in viscosity of the different chitosan/GP samples as a function of time at 37 °C. The chitosan-PA/GP (9:1) sample had a higher viscosity than the chitosan-PA/GP (7:1) at 15 min ($P < 0.05$). In contrast, the viscosity of the chitosan-SA/GP (9:1) changed very little when compared to the two chitosan-PA/GP (9:1 and 7:1) ($P < 0.05$). Jarry *et al.* (Jarry et al., 2001) previously demonstrated that autoclaving chitosan solution significantly affected viscosity, resulting in a 3–5-fold decrease in dynamic viscosity. In our experiments, however, autoclaving the dry chitosan power had no effect on viscosity. The viscosity of the chitosan-SA/GP (9:1) was lower than that observed for the other chitosan preparations examined. In addition, some prior studies (Hwang & Shin, 2000; Mucha, 1997) demonstrated that the viscosity of chitosan solutions increases with increasing chitosan concentration, and this effect has been shown to be due to increased entanglements that occur between the macromolecular chains. Obviously, the chitosan concentration in chitosan-PA/GP (9:1) was higher than that in chitosan-PA/GP (7:1), indicating the viscosity of chitosan-PA/GP (9:1) is higher than that in chitosan-PA/GP (7:1). Ji et al. demonstrated that the viscosity of CS-HTCC/GP was only approximately 2 Pa s, which was significantly lower than the viscosity of our chitosan-PA/GP (9:1) hydrogel (40 Pa s). A lower viscosity in the CS-HTCC/GP formulation may be because the HTCC decreased the crystallinity and improved chitosan water solubility (Wu, Wei, Wang, Su, & Ma, 2007).

Some researchers found that adding polyols to chitosan solutions could reduce the autoclave-induced viscosity loss and have a positive effect on the compressive performance of the hydrogel (Jarry et al., 2002). It was also found that γ-rays could also reduce the viscosity of chitosan, changing its thermosensitivity

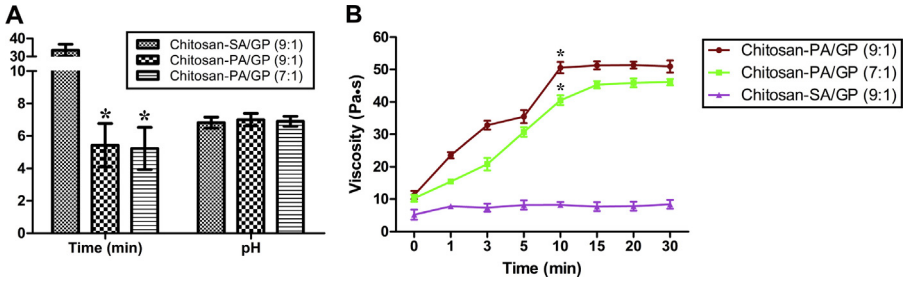

Fig. 3. Thermosensitivity and viscosity of the chitosan/GP hydrogels. (A) Gelation time and pH value of chitosan/GP hydrogels. The data are depicted as the mean ± SD ($n = 5$). *$P < 0.05$ when compared with the chitosan-SA/GP (9:1) group. (B) Plot of chitosan/GP hydrogel viscosity as a function of time at 37 °C. The chitosan-PA/GP (9:1) group (red line), chitosan-PA/GP (7:1) group (green line), and chitosan-SA/GP (9:1) group (purple line) were depicted as the mean ± SD ($n = 3$). *$P < 0.05$ at the 10 min time point for the chitosan-PA/GP (9:1 and 7:1) groups compared to the chitosan-SA/GP (9:1) group.

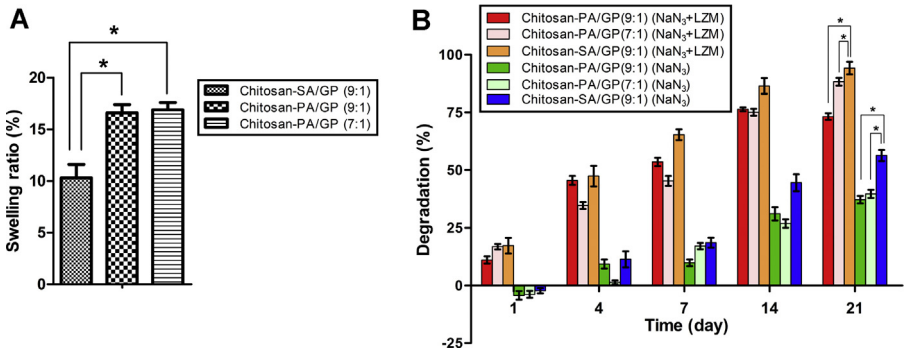

Fig. 4. Swelling degree and degradation degree of the chitosan/GP hydrogels. (A) The data are depicted as mean ± SD ($n = 5$). *$P < 0.05$ when compared with the chitosan-SA/GP (9:1) group. (B) Hydrogel degradation in different media with or without lysozyme (LZM) over a 3-week period. Data are depicted as mean ± SD ($n = 3$). *$P < 0.05$ at 21 days. *$P < 0.05$ on day 21 for the chitosan-SA/GP (9:1) group compared to the chitosan-PA/GP (9:1 and 7:1) groups.

significantly. This was predominantly due to the modest effect that addition of polyols had on inhibition of chain scission under heating conditions. A low-viscosity chitosan solution is unable to form a hydrogel in a wound once blood or tissue fluids diluted the solution further. Wu *et al.* (Wu, Su, & Ma, 2006) demonstrated that addition of glycidyltrimethylammonium chloride to chitosan solutions could increase viscosity and strength and eliminate the effects caused by the high-pressure steam. Here, we have shown that autoclaving chitosan dry powder for 10 min was sufficient to dramatically increase viscosity without the addition of other substances.

3.2. Swelling characteristics

The ability of the chitosan/GP hydrogel to swell plays a critical role in regulating nutrient and waste exchange in the hydrogel scaffold. The ability of the scaffold to absorb water aids in the storage of blood and growth factors. The degree of swelling is shown in Fig. 4a. The chitosan-PA/GP (9:1 and 7:1) samples exhibited little variation in water absorption (16–17%) and showed no statistical significance. Compared to the test groups, the chitosan-SA/GP (9:1) had a lower degree of swelling. The higher porosity of the chitosan-PA/GP (9:1 and 7:1) provided a larger surface area contact between the hydrogel and media and thus resulted in higher swelling degree.

Because chitosan contains amino groups, the addition of GP into solution allows for the formation of a charged network of ions within the hydrogel with a varying pH value. Seda *et al.* (Seda Tigli, Karakecili, & Gumusderelioglu, 2007) indicated that the amine groups were more hydrophilic than acetamide groups, but found no relationship between degree of deacetylation and the degree of swelling. A large osmotic pressure or increased pH can also cause hydrogel swelling (Chen et al., 2004). A higher concentration of mobile ions could lead to increased osmotic pressure, which would ultimately increase the degree of hydrogel swelling. Similar to other hydrogels with the ability to swell, chitosan hydrogels have a high potential for diverse tissue engineering applications (Tan, Rubin, & Marra, 2010).

3.3. Degradation characteristics

Biodegradability has been a primary research focus in the development of hydrogels for clinical use. Chitosan hydrogel is advantageous because it is believed to degrade naturally as the cells infiltrate take over. Degradation time significantly affects whether the hydrogel is able to function as a scaffold and facilitate cell growth. After incubation with lysozyme, all three groups biodegraded by approximately 20% on the first day (Fig. 4b) and there was no significant difference in the degradation rate between the three groups. Degradation gradually increased over time, though the overall rate of degradation was largely unchanged by the 21st day. The degradation properties of chitosan (substrate) will be affected by the concentrations of the enzyme present, as is the case for any enzyme-catalyzed reaction. A high concentration of degradation products, or a low concentration of enzyme in the medium, will decrease the reaction rate (Cunha-Reis et al., 2007). Therefore, we investigated the degradation on day 21 and found that the rate of degradation was largely unchanged from that observed on day 1 of the experiment. The presence of a high concentration of degradation products and a low concentration of enzyme available for substrate reaction in the residual material likely contributed to this observation. The chitosan-SA/GP (9:1) showed over 90% degradation, which was the highest compared with the other two groups ($P < 0.05$). It has been previously published that chitosan degrades faster in the presence of lysozyme (Vert, 2009). Lysozyme catalyzed the breakdown of glucosaminides on chitosan, degrading it into small polymers (Nordtveit, Vårum, & Smidsrød, 1994).

The degradation behavior of chitosan is affected by chain length, deacetylation degree, cross-linking density, pH of the degradation media, and compounding with other materials (Gorgieva & Kokol, 2012; Wei, Cai, Lin, Wang, & Zhang, 2011). Our results were in accordance with those from a previous study by Zhang *et al.* (Zhang & Neau, 2001) who demonstrated that a lower MW equated to a faster degradation rate. Our studies of the chitosan-SA/GP (9:1) also showed that the steam-sterilized chitosan solutions had a reduced MW (Jarry et al., 2001), which caused more degradation than observed for the chitosan-PA/GP (9:1 and 7:1) preparations. Other researchers (Ganji, Abdekhodaie, & Ramazani, 2007) have previously demonstrated that chitosan hydrogels with lower chitosan concentration have faster degradation rates than those with higher chitosan concentrations. In our study, the degradation rate of chitosan-PA/GP (7:1) was faster than that of the chitosan-PA/GP (9:1), the latter of which had higher chitosan concentration. Overall, the chitosan-PA/GP (9:1 and 7:1) hydrogels showed less degradation than the chitosan-SA/GP (9:1) hydrogel.

3.4. MTT assay

After 1 day of culture, the cells began to adhere to the hydrogel. HPDLCs proliferation increased over time in the three test groups. We observed no difference in proliferation between the four groups ($P > 0.05$) (Fig. 5). This is similar to what has previously been reported that different concentrations of CS hydrogel promoted proliferation and showed good biocompatibility (Ji, Yu, Xu, & Wu, 2012). Park *et al.* (Park et al., 2003) found that chitosan enhanced the formation of new alveolar bone and cementum. Some studies also showed that chitosan could enhance the migration and

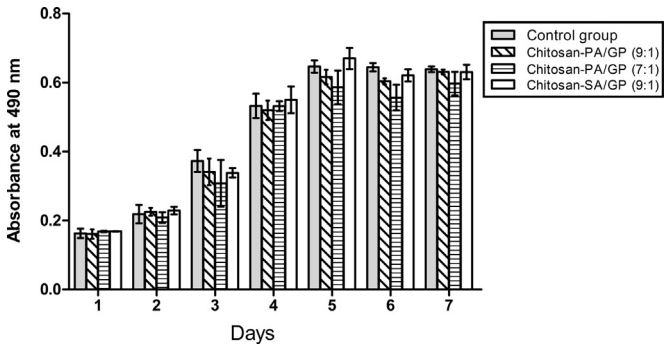

Fig. 5. Proliferation–time curve of HPDLCs chitosan/GP hydrogel extract measured by MTT assay (mean ± SD; $n = 5$).

differentiation of osteoblasts and HPDLCs (Pang et al., 2005). Chitosan has also had clinical applications as a bone substitute and as a scaffold for cell attachment and potentially for growth factors as well (Zhang, Ni, Zhang, & Ratner, 2003).

3.5. Cell attachment and staining

In order to observe HPDLC infiltration and proliferation in the chitosan hydrogel, we stained cells with DAPI at 1 day and 7 days in culture. The chitosan hydrogel maintained good cytocompatibility (Fig. 6a), which was further supported by the quantitative results of the MTT assay. DAPI staining also showed an increase in the number of HPDLCs attached to and proliferating within the chitosan-PA/GP (9:1 and 7:1) hydrogels and chitosan-SA/GP (9:1).

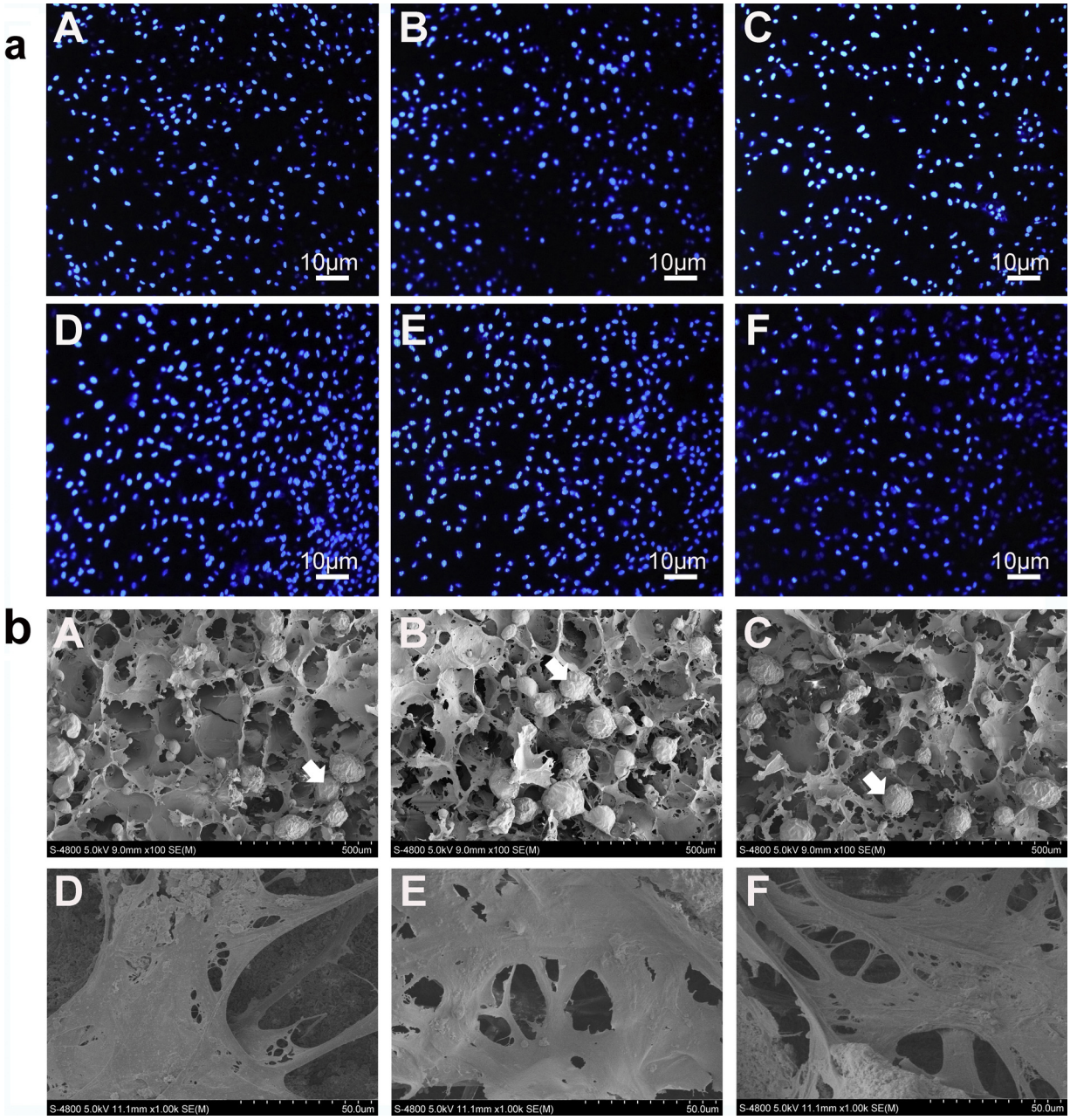

Fig. 6. HPDLCs adhesion and proliferation in the chitosan/GP hydrogels. (a) Fluorescent micrographs of HPDLCs labeled with DAPI. HPLDCs were seeded on (A, D) chitosan-SA/GP (9:1), (B, E) chitosan-PA/GP (9:1), or (C, F) chitosan-PA/GP (7:1) hydrogel and examined on culture days 1 (A–C) and 7 (D–F). (b) SEM images depicting morphology and attachment of HPDLCs in the (A, D) chitosan-SA/GP (9:1) group, (B, E) chitosan-PA/GP (9:1) group, and (C, F) chitosan-PA/GP (7:1) group. (A–C) Day 1 of culturing (×100). (D–F) Day 7 of culturing (×1000). Arrows point to the HPDLCs on the chitosan/GP hydrogel.

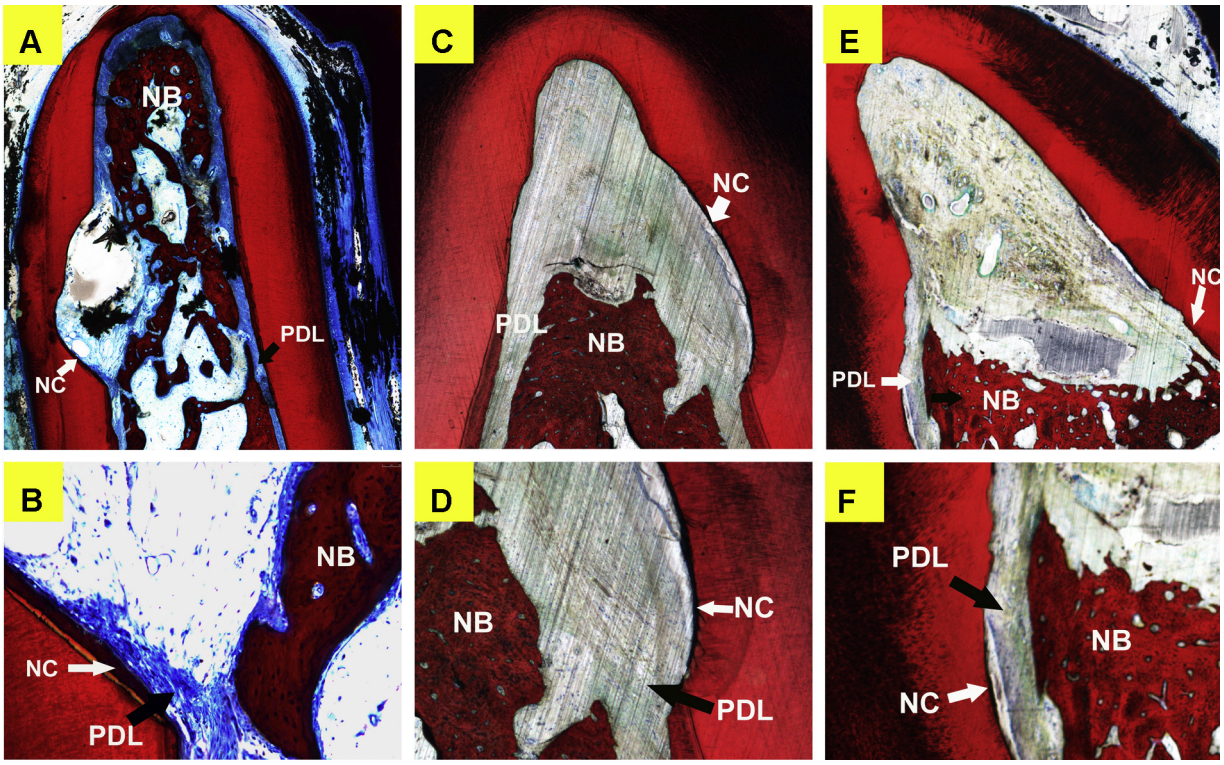

Fig. 7. Histological observations in the class III furcation defects of a canine model. (A, B) In the chitosan-PA/GP (9:1) group, the defect was occupied by newly formed bone with a few connective and epithelial tissues present. New bone containing osteoblasts, cell-like newly formed cementum (NC) (white arrowheads) and newly regenerated periodontal ligament (PDL) (black arrowheads) were observed. (C, D) In the chitosan-SA/GP (9:1) group, the defects were partially filled in with newly formed bone, but to a lesser extent than the chitosan-PA/GP (9:1) group. (E, F) In the negative control group, the defect was predominantly occupied by connective tissue. Less bone formed in the controls in comparison to the other two groups. NC (white arrowheads) and PDL (black arrowheads) could be observed in the three groups. NB: new bone formation; PDL: periodontal ligament regeneration; NC: new cementum formation; VG: Van Gieson's staining. A, C, D: ×25, B, D, F: ×100.

Analysis using SEM revealed the cellular morphology and cell attachment on the scaffold surface (Fig. 6b). After 1 day of cell incubation, we found that HPDLCs adhered well and displayed a normal morphology on the hydrogel surface. After 7 days in culture, the cells changed from a rounded shape to a flattened and spread morphology, covering a majority of the hydrogel surface. This may have been a consequence of the increased surface area and porous nature of the hydrogel. It was well known that increasing pore size was associated with good cell penetration (Whang et al., 1999). In our study, the average pore diameter of our three groups was 177.2 ± 37.2 μm, which aligns well with work from previous studies. Ji *et al.* (Ji et al., 2011) demonstrated that fibroblasts could penetrate up to 400 μm in depth into hydrogel with reasonable viabilities when hydrogels had larger pores (>90 μm) and high porosities (>85%).

The MTT assay was used to evaluate the effects of the chitosan/GP thermosensitive hydrogel on the viability of HPDLCs. In some previous studies (Ahmadi & de Bruijn, 2008; Ji et al., 2009b), the chitosan/GP thermosensitive hydrogel was soaked in culture medium at 37 °C for 24 h; the extracts found in the culture medium contained almost all of the soluble compounds of the hydrogel and cytotoxicity over a 7-day period was measured by the MTT assay. The concentration of soluble compounds from the hydrogel may be changed at day 7 of the experiment. Investigation of cell viability, distribution, and morphology on the chitosan/GP hydrogel in the current study, using fluorescent inverted microscopy assay and SEM, showed that the cells cultured with chitosan/GP hydrogel for 7 days were able to exert a continuous response to the all of the soluble compounds of the chitosan/GP hydrogel. Thus, cells cultured in chitosan/GP hydrogel may have a different biocompatibility, which may cause the cell–hydrogel interactions to be different from those measured by MTT assay. However, we found no significant difference among the three groups when we analyzed them by DAPI staining on day 7, which was in accordance with the results we obtained using the MTT assay.

The results presented in Fig. 6b indicate that the cells were able to adhere to the scaffolds regardless of the pore diameter and porosity. The long-term viability of this extended-surface adhesion property is currently being investigated in a related research project in our laboratory.

3.6. Repair of periodontal defects in a canine model

Increasing the viscosity of an injectable graft can promote the operative convenience and stabilize clotting to promote the success of a periodontal regeneration procedure. In addition, a suitable degradation time is needed for successful periodontal regeneration, one that is adequate in length to facilitate storage of cells and growth factors for adequate availability to support the biological process of regeneration. Based on the results from experiments described above, the chitosan-PA/GP (9:1) hydrogel exhibited higher viscosity and lower degradation than the chitosan-PA/GP (7:1) hydrogel, suggesting that the chitosan-PA/GP (9:1) preparation was more suitable for use as an injectable graft in periodontal tissue engineering, even though the chitosan-PA/GP (9:1) and chitosan-PA/GP (7:1) preparations showed no significant differences in gelation time, swelling time, or biocompatibility properties.

We selected the chitosan-PA/GP (9:1) hydrogel for comparative analysis against the chitosan-SA/GP (9:1) hydrogel, which was carried out using the canine model of periodontal regeneration. After addition of either hydrogel, we observed no inflammation.

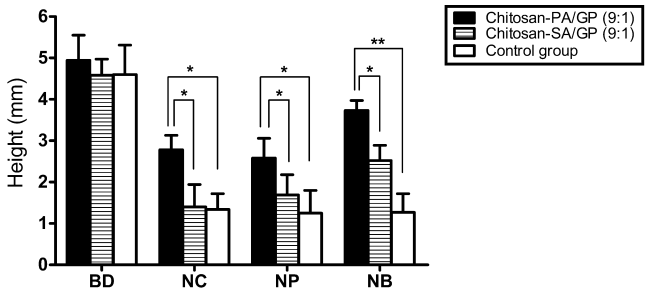

Fig. 8. Histometric parameters for canine tissues treated with the various hydrogels. The data are depicted as the mean ± SD ($n = 8$). $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to the chitosan-SA/GP (9:1) group. BD: bone defect height; NB: new bone formation; PDL: periodontal ligament regeneration; NC: new cementum formation.

Tissue specimens were stained using the Van Gieson's staining method (Fig. 7). In the chitosan-PA/GP (9:1) hydrogel group, approximately 80% of the furcation area was filled with new bone. In the chitosan-SA/GP (9:1) group, new bone only comprised approximately 30% of the furcation area. Few periodontal tissues (e.g., periodontal ligament regeneration (PDL), new cementum formation (NC), new bone formation (NB)) were observed in the negative control group. The values of NB in the chitosan-PA/GP (9:1) and chitosan-SA/GP (9:1) hydrogel groups were significantly higher than those in the negative control group (Fig. 8). The NC and NP levels were essentially the same among the groups.

Periodontal tissue engineering using scaffolds has garnered considerable attention and made significant progress over the past decades. Class III furcation defects, however, have proven challenging due to the lack of anatomical factors for clot stabilization and protection. Previous reports have shown limited regeneration in a class III furcation defect model (Jiang et al., 2010; Suaid et al., 2012). Here, the chitosan-PA/GP (9:1) group showed a dramatic increase in bone regeneration (approximately 80%) when compared with the other groups. This increased bone growth may be attributed to stimulation by the gel. In contrast to the results obtained by Ji *et al.*, our histological analysis demonstrated a significant degree of periodontal tissue regeneration in the experimental group, without co-administration of growth factors. Hydrogels have several different functions in periodontal tissue engineering. Hydrogels function as three-dimensional scaffolds that fill the defect while providing space for cell adhesion, proliferation, and differentiation. They also serve as delivery vehicles carrying cells, drugs, or bioactive molecules (Drury & Mooney, 2003; Khor & Lim, 2003). When previous investigators (Murano et al., 2006; Suaid et al., 2012) used the same periodontal defect model, they also observed a larger bone growth area (84.8%) in the group treated with PDL cells, as compared with the control group (12.2%). Further experiments will be needed to study the effect of the cells on this process.

4. Conclusions

In our study, the chitosan-PA/GP (9:1 and 7:1 volume ratio of chitosan and GP) hydrogels were prepared by first autoclaving the chitosan power and the physicochemical properties and biocompatibility characteristics of these preparations were then compared to those of the traditionally prepared chitosan-SA/GP hydrogel (autoclaving chitosan solution). The chitosan-PA/GP (9:1) hydrogel maintained its thermosensitivity and stayed in an aqueous solution below 25 °C, only turning into a hydrogel after reaching 37 °C. The hydrogel had a 140–230 μm pore size, proper viscosity, swelling ability, and limited degradation. It also demonstrated good biocompatibility characteristics and enhanced periodontal ligament cell proliferation *in vitro*. The chitosan-PA/GP (9:1) thermosensitive hydrogel promotes periodontal tissue regeneration and can be used as a tissue engineering scaffold. Future research will test the immunological response of host cells toward the hydrogel *in vivo*.

Acknowledgments

This study was funded by a grant from the National Natural Science Foundation of China (No. 81271137).

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