

The effects of intro-oral parathyroid hormone on the healing of tooth extraction socket: an experimental study on hyperglycemic rats

Abstract

Lin XU¹ 
 Li MEI²
 Rui ZHAO³
 Jianru YI¹
 Yixuan JIANG⁴
 Ruomei LI⁵
 Youliang ZHAO⁶
 Li PI⁷
 Yu LI¹

Objective: To investigate the effects of intro-oral injection of parathyroid hormone (PTH) on tooth extraction wound healing in hyperglycemic rats. **Methodology:** 60 male Sprague-Dawley rats were randomly divided into the normal group (n=30) and DM group (n=30). Type 1 diabetes mellitus (DM) was induced by streptozotocin. After extracting the left first molar of all rats, each group was further divided into 3 subgroups (n=10 per subgroup), receiving the administration of intermittent PTH, continuous PTH and saline (control), respectively. The intermittent-PTH group received intra-oral injection of PTH three times per week for two weeks. A thermosensitive controlled-release hydrogel was synthesized for continuous-PTH administration. The serum chemistry was determined to evaluate the systemic condition. All animals were sacrificed after 14 days. Micro-computed tomography (Micro-CT) and histological analyses were used to evaluate the healing of extraction sockets. **Results:** The level of serum glucose in the DM groups was significantly higher than that in the non-DM groups ($p < 0.05$); the level of serum calcium was similar in all groups ($p > 0.05$). Micro-CT analysis showed that the DM group had a significantly lower alveolar bone trabecular number (Tb.N) and higher trabecular separation (Tb.Sp) than the normal group ($p < 0.05$). The histological analyses showed that no significant difference in the amount of new bone (hard tissue) formation was found between the PTH and non-PTH groups ($p > 0.05$). **Conclusions:** Bone formation in the extraction socket of the type 1 diabetic rats was reduced. PTH did not improve the healing of hard and soft tissues. The different PTH administration regimes (continuous vs. intermittent) had similar effect on tissue healing. These results demonstrated that the metabolic characteristics of the hyperglycemic rats produced a condition that was unable to respond to PTH treatment.

Keywords: Diabetes mellitus. Tooth extraction. Parathyroid hormone. Wound healing.

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Corresponding address:
 Professor Yu Li
 State Key Laboratory of Oral Diseases - National
 Clinical Research Center for Oral Diseases -
 Department of Orthodontics - West China Hospital of
 Stomatology - Sichuan University,
 No.14, South Renmin Road - section 3 -
 Chengdu - 610041 - China.
 Phone: (+86) 028-85503645
 e-mail: yuli@scu.edu.cn

¹State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Orthodontics, West China Hospital of Stomatology, Sichuan University, Chengdu, China.

²Discipline of Orthodontics, Department of Oral Sciences, Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, New Zealand.

³State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Maxillofacial Surgery, West China Hospital of Stomatology, Sichuan University, Chengdu, China.

⁴State Key Laboratory of Oral Diseases, National Clinical Research Center for Oral Diseases, Department of Oral Implantology, West China Hospital of Stomatology, Sichuan University, Chengdu, China.

⁵Department of Orthodontics, Ninth People's Hospital, School of Stomatology, Shanghai Key Laboratory of Stomatology, Shanghai Jiao Tong University, Shanghai, China

⁶Department of emergency department, West China Second Hospital, Sichuan University, Chengdu, China

⁷West China School of Public Health, Sichuan University, Chengdu, China



Introduction

Diabetes mellitus (DM) has an increasingly higher occurrence of 463 million adults worldwide.¹ People with DM are more likely to suffer from earlier detrimental oral status and are prone to numerous oral diseases, including oral infection, periodontitis and difficulties in wound healing after tooth extraction.² It has been found that the prevalence of tooth extractions in DM patients is 1.88 times higher than the general population.³

The high-glucose microenvironment would delay the healing of tooth extraction sockets.⁴ High glucose could affect the osteoblastic function and matrix mineralization.⁵ The soft tissue repair is associated with the defect in the formation of granulation tissue and collagen degradation induced by excessive matrix metalloproteinase.⁶ The dysfunction of fibroblast could impair the collagen synthesis, resulting in decreased fiber accumulation.⁷ Many studies have explored different approaches to accelerate the healing of tooth extraction sockets under DM conditions, such as the use of growth factors,⁸ graft fillings,⁹ ellagic acid,¹⁰ and low-level laser therapy.¹¹ The effectiveness of these approaches, however, is unsatisfactory due to the complexity of DM and vulnerability of the oral environment.¹²

Parathyroid hormone (PTH) is one of the important hormones to maintain the balance of the calcium and phosphorus metabolism.¹³ PTH can regulate osteoclast activity and influence bone remodeling, and is currently the only clinical drug to promote osteogenesis for the treatment of osteoporosis.¹⁴ The intermittent administration of PTH has been found to directly inhibit transcription of the sclerosis gene and stimulate bone formation;¹⁵ the continuous administration of PTH could increase the receptor activator of the nuclear factor- κ B ligand/osteoprotegerin ratio, resulting in an increased osteoclastogenesis.¹⁶ PTH could also benefit the healing of soft tissue via suppressing inflammation and stimulating collagen deposition.⁴ A recent study has reported that PTH promoted the healing of both hard and soft tissues in the tooth extraction socket.¹⁷

The effect of PTH on extraction socket healing under DM condition remains unclear. The aim of this study was to investigate and compare the effects of local administration (intro-oral injection) of intermittent- and continuous-PTH on the healing of tooth extraction sockets in hyperglycemic rats.

Methodology

Synthesis and characterization of PTH controlled-release hydrogel

A thermosensitive and injectable hydrogel was prepared for the continuous administration of PTH based on the literature.¹⁸ The poly (ethylene glycol)-poly (caprolactone)-poly (ethylene glycol) thermosensitive hydrogel (PEG-PCL-PEG, PECE) was synthesized by ring-opening copolymerization.¹⁹ The ¹H Nuclear Magnetic Resonance (¹H NMR) spectra (in CDCl₃) was recorded on a Varian 400 spectrometer (Varian, USA) at 400 MHz to characterize chemical composition of PECE copolymers.

The sol-gel-sol phase transition behavior was carried out as follows: PECE hydrogel was placed into 5 mL EP tube and incubated in a water bath at 0°C for ten min, and then was slowly heated at a rate of 0.5°C/min until 37°C. The sol-gel-sol phase transition diagram was recorded using the test tube-inverting method, which was visually observed by inverting the tube. The condition of sol and gel phase was defined as "flow liquid sol" and "no flow solid gel".

In vitro release tests at body temperature were carried out as follows: firstly, 750 μ L hydrogel loaded with 1 mg BAS protein was placed into the bottom of the EP tube at 37°C for 1 h to form the gel. Then, 1000 μ L Milli-Q water was added into the tube to immerse the gel. Then, the sample was kept and shaken at 106 rpm at 37°C. The solution was collected at each time of interest (T=0, 1, 2, 4, 8, 12, 24 h in the first day, and at each 24 h interval until 336 h) and replaced with 1000 μ L preheated fresh Milli-Q water. After centrifugation at 13,000 rpm for 10 min, the measured extracts were stored at -20°C for drug release analysis. The amount of released protein at each time points was assessed in triplicate using the Enhanced BCA Protein Assay kit (Beyotime technology, Beijing, China) following manufacturer's instructions. The accumulative release percent of BSA protein was calculated.

Animal model and tooth extraction

A total of 60 young male Sprague-Dawley rats (5 weeks old) were included in the study. Sample size was calculated by power analysis based on the estimated effect size reported in the previous study.²⁰ Rats were housed at 22 °C, 40% humidity, and in 12-hour daylight cycles. Rats were allowed access to standard laboratory rodent diet and water. The ethics

of the study was approved by the Research Ethics Committee of West China Hospital of Stomatology (No. 201610610379). The study was presented following the Animal Research N3CRs guidelines for Reporting of In Vivo Experiments (ARRIVE) guidelines.

Sixty rats were randomly divided into two groups (n=30 per group), including the normal control and the diabetic group. Diabetes was induced into 12h-fasted rats by intraperitoneally streptozotocin (STZ) (Sigma, St Louis, MO, USA) dissolved in citrate buffer (pH=4.2-4.5). STZ was calculated at 45 mg/kg.²¹ Three days after the STZ injection, blood was obtained from the tail vein, and glucose levels were measured using a glucometer (Yuwell, Jiangsu, China). Diabetes was confirmed twice by the presence of random blood glucose concentrations of >16.7 mmol/L.¹⁰

The mandibular left first molar was extracted under general anesthesia (10% chloral hydrate solution, 0.32ml/100g) from each animal. Sterile dental instruments were used during the extraction of molars, and teeth removal was performed by using a sharpened dental explorer.²² Alveolar sockets were left to heal without sutures.

Grouping and administration of PTH

After tooth extraction, the two groups were divided randomly into 3 subgroups (n=10 per group), including the normal saline (NS), the intermittent-PTH (iPTH) and the continuous-PTH group (cPTH).

The intermittent intra-oral injection of PTH (1-34) (Chinese Peptide, Hangzhou, China) was administered at a dose of 80 µg/kg three times a week for 14 days.^{17, 23} It was dissolved in normal saline solution, and the needle was placed in the buccal vestibule next to the tooth extraction site.

The continuous intra-oral injection of PTH (1-34) was administered as follows: PECE was mixed and dissolved completely in NS to form sol in the temperature of 55-60°C at a concentration of 25 wt% and cooled at 0 °C for three minutes.¹⁹ Then, PTH (1-34) solution was mixed into the PECE sol to form suspension, and the PTH (1-34) gel to release slowly for 14 days was obtained. Soon after the tooth extraction, PECE hydrogel loaded with an equivalent dosage of PTH (1-34) in iPTH group was put in the tooth socket.

Animals in the two NS groups (NS normal group and NS diabetic group) were injected with the same volume of normal saline solution three times a week

for 14 days.

Serum chemistry

Blood was collected from carotid artery at the time of euthanasia, which was 24 h after the last injection of PTH. Serum samples were prepared and used immediately. The levels of calcium were measured using a commercial kit (Jiancheng, Nanjing, China). The blood glucose levels from the tail vein of each group were monitored once every week using a glucometer (Yuwell, Jiangsu, China). Diabetic rats with unsatisfactory serum glucose levels would be removed; however, no animal was excluded for this reason.

Micro computed tomography (Micro-CT) assessment

Rats were euthanized at the end of the designated injection period of 14 days. The mandibles were dissected, and fixed in 10% formalin, and the extraction wounds were scanned by Micro-CT (Micro-CT50, Scanco Medical, Bassersdorf, Switzerland) at 10 µm voxel resolution with the beam energy level of 70 kVp and electrical current of 0.2 mA. The time of scanning was 858 ms. Each mandible was fixed by upside-down styrofoam, and the occlusal plane became horizontal to the bottom. The conservation of the samples occurred in 10% formalin. The thresholds were determined visually in several slices. Trabecular parameters were identified by the direct measurement technique. The region of interest was a trabecular compartment of 100 slices around extraction sockets and was segmented by semi-manual contour. Alveolar bone trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and bone mineral density (BMD) were analyzed with built-in software.

Histomorphometric analysis

Rats were decapitated two weeks after the interventions. The specimens were demineralized in 10% ethanol, paraffin-embedded, and sectioned to 5 µm thickness. Hematoxylin and eosin (HE) (Beyotime technology, Beijing, China) and Masson's trichrome (Sigma, St Louis, MO, USA) staining techniques were performed for the detection of bone tissue and collagen fibers respectively, following the manufacturer's instructions. The staining sections were analyzed by Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). New alveolar bone formation was quantified in

the extraction wounds as described previously.²⁰

Statistics analysis

Statistical analyses were performed using the SPSS statistics base 17 (SPSS Inc., Chicago, IL, USA). Results are presented as mean±standard deviation. The normality test was performed using the Shapiro-Wilk analysis; the statistical comparisons were analyzed by the Student's t-test, one-way ANOVA, and two-way ANOVA, with a significance level of 5%. Statistical significance was defined as 0.05 α -level.

Results

Characterization of PTH controlled-release hydrogel

The chemical structure of PECE gel was characterized by ¹H NMR (Figure 1). The peak of the caprolactone unit and the ethylene glycol unit were at 3.98 ppm and 3.57 ppm, respectively. The macromolecular weight estimated from ¹H NMR spectrum was 3246, which was consistent with theoretical estimation, indicating that the PECE gel was synthesized successfully.

The temperature-dependent sol-gel phase transition behavior was presented in Figure 2A. The hydrogel showed a sol state at lower temperature (0°C) and a gel state at body temperature (37°C). The hydrogel underwent sol-gel-sol phase transition as the temperature increased and slowly released PTH at local part, which prolonged the therapeutic effect time and reduced the repeated injections.

The controlled-release ability of PECE hydrogel

is shown in Figure 2B. The release rate of the drug was rapid in the first 168 hours, and then became comparatively slow and sustained in the next 168 hours. The cumulative release of protein reached 58.24% by the 14th day. Therefore, the experiment period for the PTH controlled-release hydrogel was set as 14 days in this study.

Serum chemistry

The level of serum glucose in the DM groups (DM+NS, DM+iPTH and DM+cPTH) (mean 22.38±2.71 mmol/L, with no significant difference within the three groups, $p>0.05$) was significantly higher than the non-DM groups (NOR+NS, NOR+iPTH and NOR+cPTH), (mean 5.82±0.67 mmol/L) ($p<0.01$), indicating that the DM model was successfully established in the study.

No statistical significance of the serum calcium concentration was found either between the DM and non-DM groups, or within these groups ($p>0.05$ for all). Local administration in the extraction socket in the study could reduce the systematic effects of PTH, such as hypercalcemia, which was reflected by the unaltered serum calcium level.

Micro-CT analysis of the extraction socket

The Micro-CT analysis (Figure 3) showed that the Tb.N in NOR+NS group ($2.37 \pm 0.29 \text{ mm}^{-1}$) was significantly higher than that in DM+NS group ($1.52 \pm 0.14 \text{ mm}^{-1}$) ($p<0.01$) (Figure 1E), while the Tb.Sp in NOR+NS group ($0.21 \pm 0.09 \text{ mm}$) was significantly lower than that in DM+NS group ($0.47 \pm 0.05 \text{ mm}$) ($p<0.05$) (Figure 1F), indicating that bone density was affected by the DM condition. No significant difference

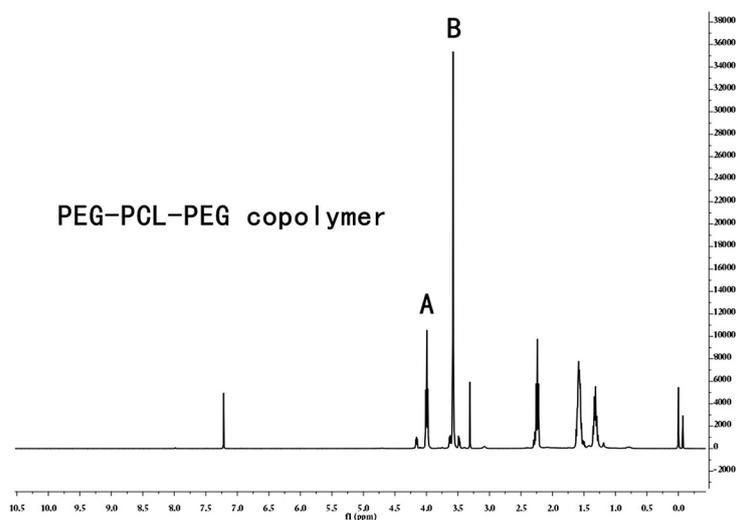


Figure 1- ¹H NMR spectrum of PECE copolymer. The peak of (A) caprolactone unit and (B) ethylene glycol unit were at 3.98ppm and 3.57ppm, respectively

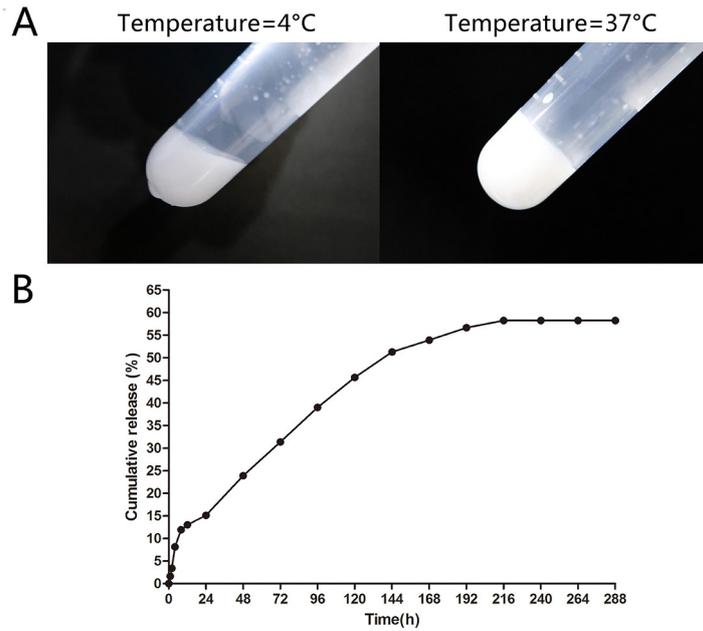


Figure 2- Characterization of PTH controlled-release hydrogel (A) The sol-gel phase transition behavior of PECE hydrogel: the solution state of hydrogel at 0°C and the gel state of hydrogel at 37 °C. (B) The cumulative release of drug-loaded PECE hydrogel for 14 days

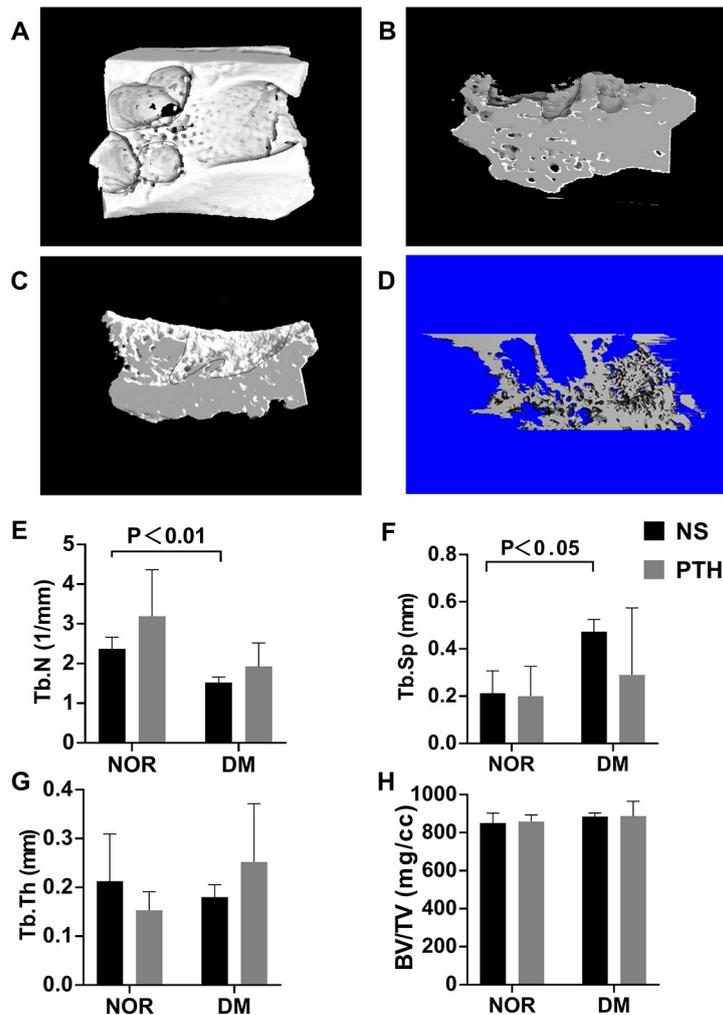


Figure 3- Micro-CT analysis of the extraction socket. (A) Cross section of the Micro-CT scan in the extraction socket; (B) Sagittal plane of the Micro-CT scan in the DM+iPTH group; (C) Sagittal plane of the Micro-CT scan in DM+NS group; (D) 3D reconstruction of the extraction socket in DM+PTH group. (E), (F), (G) and (H) Micro-CT analysis of Tb.N, Tb.Sp, Tb.Th and BMD. Tb.N in NOR+NS group was significantly higher than that in DM+NS group ($p < 0.01$), while the Tb.Sp in NOR+NS group was significantly lower than that in DM+NS group ($p < 0.05$). PTH therapy for 14 days induced no detectable bone anabolic effect in alveolar socket bone parameters

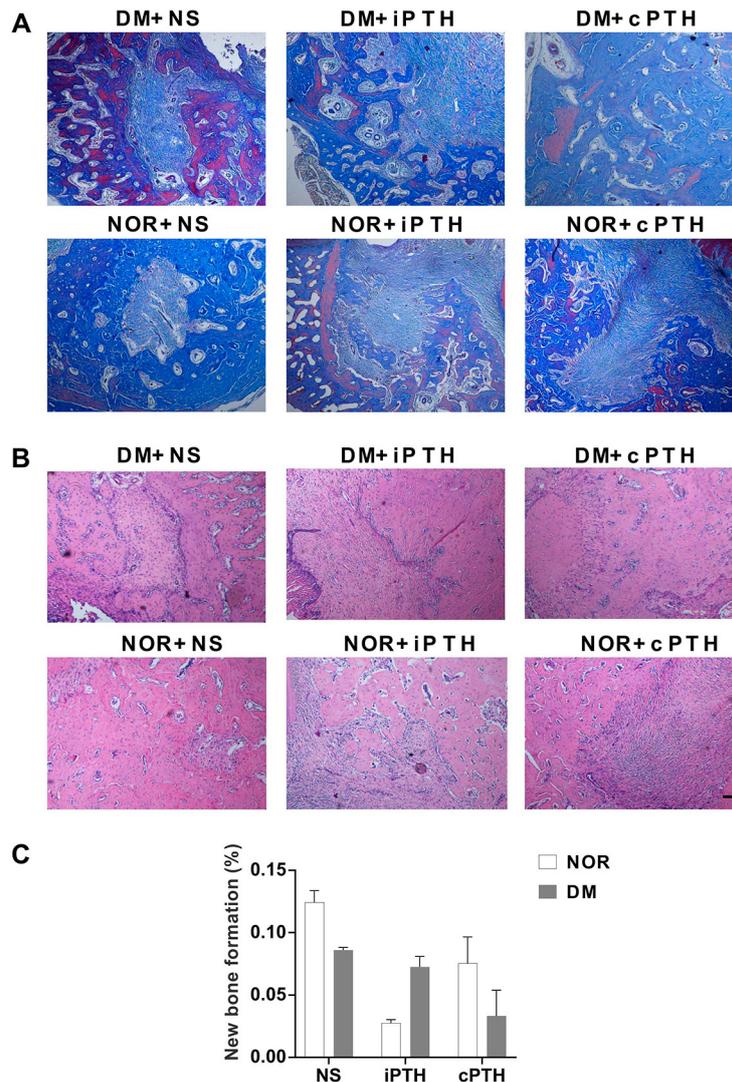


Figure 4- Histological analysis of the extraction socket. (A) Masson's trichrome staining (red parts meant new bone and light blue parts meant collagen. Scale bar: 100µm); (B) HE staining; and (C) New alveolar bone formation in the tooth extraction socket. PTH therapy for 14 days induced no detectable new alveolar bone formation

was found among all the other groups for the alveolar bone Tb.Th and BMD ($p > 0.05$ for all). Bone formation in the extraction socket under the DM conditions in the study was significantly less than that under non-DM condition, indicating an impaired hard-tissue healing of extraction socket under DM condition.

There is no significant difference of Tb.N, Tb.Th, Tb.Sp and BMD between the PTH and NS groups ($p > 0.05$ for all), indicating that the effect of PTH on tooth extraction socket in normal and DM rats was minimal (Figure 2 E-H).

Histological analysis of the extraction socket

The histological analysis of soft tissue (HE and Masson's trichrome staining) showed that more collagen in the extraction socket was observed in the groups treated with PTH (both iPTH and cPTH) than those not treated with PTH (Figure 4A and B). The

quantitative analysis of hard tissue (new alveolar bone formation) showed no statistical significance between DM and non-DM groups or between NS and PTH groups ($p > 0.05$) (Figure 4C).

Discussion

In this study, bone formation in the extraction socket in the DM rats was decreased when compared to that of the normal control rats. PTH (1-34) showed no obvious results in hard tissue formation and soft tissue healing. No difference was found on the healing of extraction socket between intermittent and continuous administration of PTH. These findings suggest that hyperglycemic rats failed to benefit from the anabolic treatment of PTH on the healing of extraction socket in the high glucose condition.

The effectiveness of PTH on promoting the healing of tooth extractions in the diabetic rats was not significant in the study. This may be due to the metabolic characteristics of hyperglycemic rats leading to a condition of ineffective PTH treatment. Some studies showed that PTH did not increase the insulin-immunoreactivity of β -cells of the pancreatic islets in diabetic rats.²⁴ The inflammation caused by diabetes would disrupt vascular endothelial cell function.²⁵ The expression of inflammatory cytokines such as TNF- α , IL-1 and IFN- γ in bone increased,^{26,27} which contributed to the delayed healing of extraction sockets. Moreover, the high level of advanced glycosylation end products and collagen cross-linking may cause an overall negative effect on diabetic bone mechanics.²⁸ Therefore, metabolic control of hyperglycemia may be a key factor when patients with diabetes receive anabolic therapy to improve bone mass. Future studies could explore the anabolic property of PTH in the DM condition when hyperglycemia is controlled with insulin, and immunolabeling or molecular analyses could be performed to further evaluate the possible anabolic activity of PTH and signaling pathways.

The histological analyses in the study showed that the PTH treatment showing more collagen formation demonstrated an anabolic effect on soft tissue healing. In response to hyperglycemia, advanced glycosylation end products were induced and covalently bounded to reactive amino groups, resulting in a dysfunction of the epithelial cells and extracellular matrix.⁷ This is coincident with the decreased collagen in the extraction socket in the DM rats. This may be due to the microcirculation defect and insufficient oxygen caused by thickened and inelastic vessel wall.²⁹

Several studies have shown double effects of PTH (1-34) – anabolic when administered intermittently and catabolic when applied continuously.³⁰ The subcutaneous and intermittent administration of PTH facilitated the hard and soft tissue repair in tooth extract socket at the dose of 80 μ g/kg daily.⁴ Intermittent and systemic PTH administration reduced alveolar bone loss by decreasing the receptor activator of nuclear factor kappa B ligand (RANKL)/osteoprotegerin (OPG) ratio in hyperglycemic rats.²³ However, in the continuous administration of PTH, the expression of Phospho1 and Smpd3 in osteoblasts decreased,¹⁶ resulting in the suppression of bone formation mediated by Cox2.³¹

Due to the biological nature of hard-tissue healing

in the tooth extraction socket,³² this study mainly focused on the effects of PTH on bone formation rather than resorption. However, hyperglycemic rats failed to benefit from the anabolic treatment. The contradictory action of PTH might be caused by double effect on bone formation and bone resorption. The anabolic role of iPTH could be lessened by the catabolic effect of DM.³³ At the early stage of bone and cartilage healing process, both administrations of PTH could promote osteogenic differentiation.³⁴ The cPTH did not suppress osteogenesis, but the catabolic role on bone metabolism was greater than its anabolic role, resulting in bone loss. The cPTH increased slightly undifferentiated mesenchymal stem cell, osteoblast and osteocyte number at 14 days.³⁵ This may result in the non-significant difference between two regimes of PTH. Another possible reason might be that the rats used in the study were male. The cPTH was found to be not catabolic in male rats, as no change was found in BMD and cortical thickness in males.³⁶

The experiment period adopted in this study was two weeks. This was in consideration of the optimal slow-release effect of the PECE hydrogel. Furthermore, the epithelialization was generally complete and well-keratinized at approximately two weeks.^{37, 38} The maximum bone formation was achieved and the woven bone completely filled the extraction socket,⁴ and almost replaced by substance with a radio-opacity during that timeframe,³⁷ so 14 days was appropriate for this study. The long-term effects of PTH on the healing of extraction socket under hyperglycemic conditions need further investigation.

Conclusion

Bone formation in the extraction socket under the DM condition was reduced. Hyperglycemic rats failed to benefit from the PTH treatment in the healing of tooth extraction sockets. The different PTH administration regimes (intermittent vs. continuous) had similar effects on the tissues healing.

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Conflict of interest

All authors have no conflict of interest.

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Authors' Contributions

Xu, Lin: Conceptualization (Equal); Data curation (Equal); Writing-original draft (Equal); Mei, Li: Conceptualization (Equal); Methodology (Equal); Writing-review & editing (Equal); Zhao, Rui: Data curation (Equal); Software (Equal); Yi, Jianru: Writing-review & editing (Equal) Jiang, Yixuan: Data curation (Equal); Software (Equal); Li, Ruomei: Formal analysis (Equal); Resources (Equal); Zhao, Youliang: Data curation (Equal); Pi, Li: Conceptualization (Equal); Methodology (Equal); Software (Equal); Li, Yu: Funding acquisition (Equal); Project administration (Equal); Supervision (Equal); Writing-review & editing (Equal)

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