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Inhibitory effect of nicotine on bone regeneration in mandibular distraction osteogenesis

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1. ABSTRACT

Nicotine is the main chemical component in tobacco products and its effect on bone healing remains controversial. Distraction osteogenesis is an endogenous tissue engineering technique which provides an excellent platform to study bone healing and regeneration. This study aims to evaluate the dose dependent effect of nicotine on bone regeneration using a rabbit model of mandibular distraction osteogenesis. Twenty New Zealand white rabbits were randomly assigned to four groups: sham control, placebo control, low dose nicotine (0.75g) and high dose nicotine (1.5g). 60-day time release nicotine pellets or placebo pellets were implanted in the neck subcutaneous tissue of the rabbits one week before osteotomy was performed. Then after three day latency, eleven day active distraction and four week consolidation, the animals were sacrificed and subjected to examinations by radiography, micro-computed tomography and histological analysis. The significantly lower bone volume and appearance of chondrocytes in the high dose nicotine group indicated that the bone regeneration of distraction osteogenesis was compromised by high dose nicotine exposure.

2. INTRODUCTION

Distraction osteogenesis is a method of generating new bone directly from the osteotomy site by a controlled mechanical traction device. It is the latest endogenous tissue engineering technique in reconstructive surgery, which is capable of producing a large quantity of living bone by a single stage operative procedure without additional bone grafts. When compared with the conventional orthognathic surgery, distraction osteogenesis is less invasive and is more stable. (1) Since the first clinical report on the use of distraction osteogenesis to lengthen the human mandible in 1992 (2), it has gained popularity in the treatment of severe craniofacial deformities. Moreover, distraction osteogenesis provides an excellent platform to study the bone healing and regeneration. It shares many features of embryonic growth, neonatal long bone development, as well as normal fracture healing. (3, 4) The mechanical stimulation generated by gradual traction induces the biological response of skeletal regeneration in a cascade of bone regeneration processes, and the molecular signaling during distraction osteogenesis is amplified and prolonged as long as the mechanical traction is in progress.

Cigarette smoking is a common social problem. The negative effect of smoking on bone healing has been widely reported in scientific literature. (5-8) It is a clinical challenge to treat heavy smokers due to their compromised healing ability, particularly on the need for the reconstructive surgery. Nicotine is the main chemical component, which has been found to be of highest importance in more than 4000 of potentially toxic substances, in tobacco product. (9, 10) Heavy smokers normally have plasma nicotine levels in the range of 10-70ng/ml, while light smokers have nicotine levels less than 10ng/ml. (11) The influence of nicotine on tissue healing has been explored by various delivery systems on different animal models and the results remains conflicting. (5, 9, 12-14).

This study aims to simulate the plasma nicotine levels of heavy and light smokers by implanting different doses of time release nicotine pellets, and evaluate the correlation between plasma nicotine levels and bone regeneration using a rabbit model of mandibular distraction osteogenesis.

3. MATERIALS AND METHODS

3.1. Animal care

The rabbits were kept in a dedicated animal holding facility under veterinary supervision in the Laboratory Animal Unit of Li Ka Shing Faculty of Medicine, The University of Hong Kong. The animal experiment protocol was approved by the Committee of the Use of Live Animals for Teaching and Research.

3.2. Nicotine implantation

Twenty adult New Zealand white rabbits (9 month old, 3.4-4.0 kg) were randomly assigned to four equal groups: sham control, placebo control, low dose nicotine (0.75g), and high dose nicotine (1.5g). Nicotine pellets (60-day time release) or placebo pellets (Innovative Research of America, Sarasota, USA) were implanted in the neck subcutaneous tissue of the rabbits. Total nicotine exposure time was seven weeks.

3.3. Determination of plasma nicotine concentration

3 ml of whole blood was collected from the marginal auricular vein on week one, three and seven respectively after nicotine implantation. Nicotine extraction was performed using the methods presented by Nakajima et al. (15) The plasma nicotine concentration was assayed by high-pressure liquid chromatography (HPLC) composed of a 626 pump (Waters, Milford, USA), a 486 tunable absorbance detector (Waters, Milford, USA) set at 260nm, and a SunFireTM C18 5μm 4.6mm × 150mm column (Waters, Milford, USA). The mobile phase contained 2mM sodium dihydrogen phosphate, 1mM heptanesulfonate sodium, the pH was adjusted to 6.2 with phosphoric acid and 10% acetonitrile was added to the final solution. The flow rate was 1.0 ml/minute. Acetanilide (International Laboratory, CA, USA) was used as the internal standard. All of the reagents were HPLC grade.

3.4. Osteotomy and distraction procedures

One week after nicotine implantation, a standard procedure of mandibular body osteotomy and distraction described by Zheng & Cheung (16) was performed. Briefly, the animals were given a preoperative dose of antibiotic and analgesic (long acting oxytetracycline 30 mg/kg and buprenorphine 0.03 mg/kg), and were anaesthetised by intramuscular injection of ketamine 35 mg/kg, xylazine 5 mg/kg, and acepromazine 1 mg/kg. The skin was incised along the inferior border of the mandibular body with the rabbit's head hyper-extended. The platysma muscle was dissected and the periosteum was raised from the mandible and reflected laterally to identify the mental nerve, which was located immediately anterior to the first premolar tooth. A straight body osteotomy cut was made with a small Lindermann bur immediately anterior to the first premolar root on one side of the mandible. A custom-made bone-borne distractor was placed along a plane perpendicular to the osteotomy cut and fixed by 2-mm-diameter titanium screws. The periosteum, muscle, and skin were repositioned and closed by 3-0 sutures.

After the operation, an antibiotic (long acting oxytetracycline 30 mg/kg) was administered intramuscularly twice per week for two weeks. For pain relief, buprenorphine (0.03 mg/kg) was administered subcutaneously twice daily for ten days. Each animal remained under close observation by a veterinary technician until it regained consciousness. The clinical condition, weight and food consumption of the animals were monitored. After three day latency period, distraction was activated at 0.9mm once daily for eleven days, and all rabbits were sacrificed after four weeks of consolidation (Figure 1).

3.5. Plain radiography

Each mandibular specimen was placed on an occlusal film with the lingual side touching the film. Plain radiography was performed by an Orthoralix 9200 x-ray machine (Gendex, Des Plaines, IL) under a standard condition of 50 kV, 16 mA.

3.6. Micro-computed tomography (micro-CT)

After plain radiographic examination, the distracted tissue regenerate and a 2–5 mm section of the neighboring normal bone in the distracted mandible were harvested. The specimens were subjected to qualitative and quantitative examination by a micro-CT machine μCT20 (Scano Medical AG, Bassersdorf, Switzerland) using a previously described protocol (16). Briefly, each harvested specimen was placed into a 17-mm diameter sample holder with the sagittal plane vertical to the X-ray tube. Between 120 and 140 cross sectional scans with a slice increment of 100-μm were made for each specimen. The serial scanned images of each specimen were inspected on the computer. On each scanned image, the total area of the distraction regenerate was outlined as the region of interest (ROI). The bone volume fraction (the ratio between bone volume and total volume, BV/TV) within the ROI on each section was calculated individually and a mean value of BV/TV for the total regenerate was obtained by pooling from all the scanned sections within the distraction gap. To determine

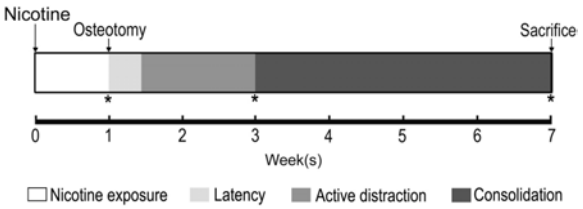

Figure 1. The time line of nicotine exposure and distraction osteogenesis. Nicotine exposure: seven weeks; latency period: three days; active distraction: eleven days; consolidation: four weeks. Plasma nicotine concentration was measured 1, 3, and 7 weeks after implantation of nicotine.

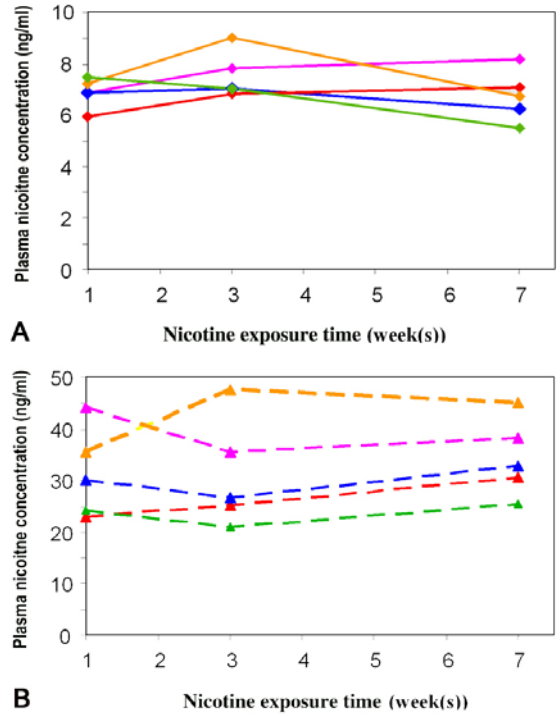

Figure 2. Plasma nicotine concentrations of the five rabbits in low dose nicotine group (A) and high dose nicotine group (B) during seven week exposure.

the threshold all ROIs of one specimen were subjected to an adaptive procedure in which the bone fraction was determined for a range of thresholds. The optimal threshold (120) was defined at the minimum change of bone fraction.

3.7. Histology

After the micro-CT examination, the samples were decalcified in a solution of 14.5% ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.2) at room temperature. The decalcified specimens were processed and embedded in paraffin wax. Axial sections of 5 µm in thickness were cut including the margin of normal bone and distraction regenerate with a microtome and stained with haematoxylin and eosin for light microscopy.

3.8. Statistical test

The differences in the values of BV/TV between four groups were compared by one way ANOVA with version 11.0 of Statistical Package for Social Sciences software (SPSS Inc., Chicago, USA). A statistical result of 0.05 was considered as significant.

4. RESULTS

4.1. Clinical examination

All twenty rabbits completed the experimental process uneventfully. None of the animals experienced postoperative complications, and distractors maintained stable till the day of sacrifice. As a result of the unilateral mandibular lengthening, the rabbits developed a severe lateral cross-bite and overgrowth of lower incisors. After the rabbit mandibles were harvested, they were confirmed to have been lengthened successfully with hard tissues filling the distraction gaps.

4.2. Plasma nicotine concentrations

In the low dose nicotine group, the plasma nicotine concentrations of five rabbits during seven week nicotine exposure were in the range of 5.47-8.15ng/ml (Figure 2A), and the average was 7.03ng/ml (SD=0.32). In the high dose nicotine group, the levels were in the range of 20.93- 47.43ng/ml (Figure 2B), and the average was 32.25ng/ml (SD=1.52). No nicotine was detected in the sham and placebo control groups.

4.3. Plain radiography

Bone union was complete and new cortex appeared partially at the margin of distraction regenerate in the control groups and low dose nicotine group (Figure 3A, 3B, 3C). In the high dose nicotine group (Figure 3D), the radiodensity in the distraction gap was lower than that in the other groups, and the height of bony regenerate was not comparable to the conjunctive host bone.

4.4. Micro-CT

The serial CT images of the distraction regenerate showed continuous ossification from the edges to the center in the distracted region. The degree of bone regenerate mineralization increased gradually from the central area to merge with the host bone at the both ends. The quantitative analysis showed that the bone volume in the distracted gap in the high dose nicotine group was significantly lower than that in the sham control ($p=0.005$), placebo control ($p=0.020$) and low dose nicotine group ($p=0.041$). There was no significant difference between the control and low dose nicotine groups (Table 1, 2).

4.5. Histology

In the control groups, complete bony union in the central area and partial corticalisation were observed in the distraction regenerate (Figure 4A, 4B). In the nicotine treated groups, the distraction area had a mixture of woven and maturing lamellar bone with a rich loose fibrovascular stroma (Figure 4C, 4D). Multiple loci of chondrocytes (Figure 4E) and small foci of fibrous tissue were noted in all the 5 rabbits in the high dose nicotine group, but were not observed in any of the 15 rabbits in the control and low dosage nicotine groups.

Table 1. Quantitative micro-CT analysis of the total area in the coronal sections of the distracted rabbit mandible

Group	Bone volume fraction (Mean ± SD[1])
Sham control	35.23 ±2.71
Placebo control	34.11 ± 2.63
Low dose nicotine	33.50 ± 2.88
High dose nicotine	28.48 ± 2.47

[1] SD: standard deviation. n=5 in each group

Table 2. One way ANOVA test of the bone volume fraction between four groups

Comparison	P value
Sham control vs Placebo control	0.911
Sham control vs Low dose nicotine	0.741
Sham control vs High dose nicotine	0.005
Placebo control vs Low dose nicotine	0.984
Placebo control vs High dose nicotine	0.020
Low dose nicotine vs High dose nicotine	0.041

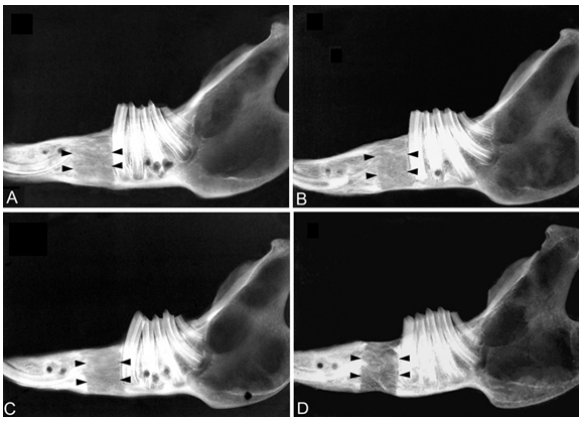

Figure 3. Lateral radiographic view of rabbit hemi-mandibles. New bone is formed in the distracted gap in the sham control (A), placebo control (B), low dose nicotine (C) and high dose nicotine (D) groups.

5. DISCUSSION

Distraction osteogenesis is an increasing popular tissue engineering technique in clinical bone reconstructive surgery. It obviates the need for taking bone grafts from patients and its associate morbidities from the donor sites and wound healing complications. Moreover, under the controlled mechanical force, distraction osteogenesis provides a convenient platform for bone research. Distraction osteogenesis consists of four clinical phases: 1. osteotomy at the target site; 2. latency period: the time gap from the osteotomy procedure to the commencement of active distraction, which allows the inflammatory phase of osteotomy healing to subside and thereby enables the distraction at the beginning of the reparative phase ; 3. active distraction: the bone fragments are separated gradually by a controlled mechanical traction at a rate of about 1mm/day till reach the proposed advancement. 4. consolidation period: the time from the complete of the active distraction till detaching the distraction device. The distraction regenerate then mineralizes and reaches the mechanical strength to achieve functional activity. The mechanical stimulation by distraction during the active distraction period induces a cascade of biologic processes of bone regeneration including differentiation of pluripotential tissue, angiogenesis, mineralization, and remodeling. When compared with bone fracture, a traditional model to study bone healing in which the molecular signaling only last a few days, the signaling in distraction osteogenesis are magnified and prolonged as long as the mechanical traction is active.

The negative effect of smoking on bone healing has been widely reported. Nicotine is the main chemical component responsible for the tobacco addition. (17) It has been found to be of highest importance among the potentially toxic substances in tobacco product (9, 10), but its influence on bone healing remains controversial. Some studies indicated that nicotine has an adverse effect on bone healing and regeneration (9, 13, 18, 19), while some other reports demonstrated no significant impact (5, 12, 14). The controversial results were likely due to the various dosages and methods of nicotine exposure and the different wound healing models used to study the nicotine influence. Thus it is important to explore a reliable and repeatable nicotine delivery method, and establish a direct correlation between nicotine plasma concentration and the bone healing.

Nicotine can be delivered by drinking water, subcutaneous injection, subcutaneous implantation, infusion by a miniosmotic pump, or smoking chambers where "research cigarette smoke" is pumped into the chamber. Orally administered nicotine is difficult to achieve the nicotine plasma levels consistent with smokers because it has to undergo a significant first-pass effect from hepatic metabolism (approximately 85-90%). (20) Subcutaneous injection is easy to handle but needs a daily injection to maintain the plasma concentration. Smoking chambers is less favorable due to the multiple agents in "smoke" versus single agent nicotine and interpretation of the results relating to nicotine is difficult. Miniosmotic pump is a reliable way for nicotine delivery, but the life span of the pump is only four weeks. (12) A secondary operation to replace the pump is needed if longer period of nicotine exposure is required. Surgical implantation of the time release nicotine pellets in the subcutaneous tissue can provide a reliable and convenient way to achieve a stable nicotine levels in a long period.

As far as we know, there is limited in vivo information on the direct correlation of plasma nicotine levels and bone healing process. We started by a conducted pilot study in our laboratory (data not shown), of which different dosages of nicotine were test whether the nicotine levels in blood can reach that of light and heavy smokers. We have selected two dosages (0.75g and 1.5g) of nicotine for the proper study. Blood samples were collected on the day of osteotomy, two weeks thereafter, and on the day of sacrifice to determine the plasma nicotine levels. The plasma nicotine concentrations in the rabbits proved to be stable during seven weeks of nicotine exposure. 0.75g nicotine pellet implantation caused a plasma nicotine level of less than 10ng/ml, which is comparable to light smokers (<1 pack/day). 1.5g nicotine implantation caused a nicotine level at 20.93- 47.43ng/ml

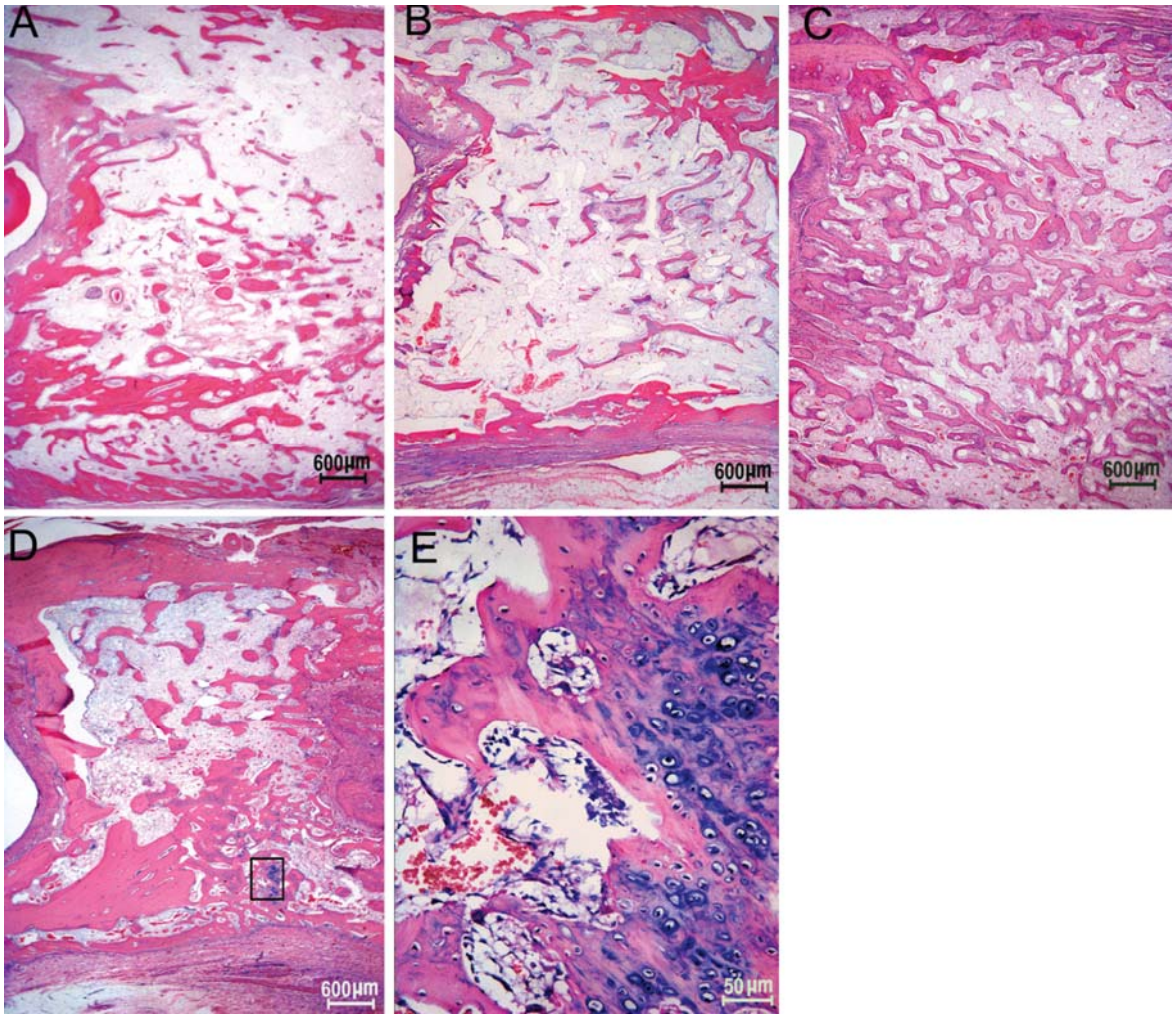

Figure 4. Histological section of the distracted regenerate in rabbit mandible (H&E stain). Complete bony union is noted in the central area of the distraction regenerate in the sham control (A) and placebo control (B) groups. A mixture of woven and maturing lamellar bone with a rich loose fibrovascular stroma are observed in the low dose nicotine (C) and high dose nicotine (D) groups. At higher magnification of the section in D, multiple loci of chondrocytes surrounded by a mineralised matrix (E) and small foci of fibrous tissue are noted.

which is comparable to heavy smokers (>1.5 pack/day). This formed the biological basis for conducting further study of the effect of nicotine on bone healing and regeneration simulating light and heavy smokers.

The present study supported that nicotine has a dose dependant influence on bone healing in distraction osteogenesis. At a low plasma level, nicotine was found to have no significant adverse effect on bone healing although the histological images showed that the newly formed bony trabeculae were not as mature as that in the control groups. This suggested that light smokers or patients who use nicotine medication for the purpose of smoking cessation (plasma nicotine concentrations are less than 10ng/ml) have little risk in bone healing in distraction. At a higher plasma nicotine concentration, the micro-CT study confirmed that the bone volume in the distraction gap was significantly less than that in control and low dose nicotine groups. This suggest heavy smokers have a considerable risk for distraction osteotomy and possibly bone reconstructive surgery.

Tissue healing processes are modulated by the activities of local cells and signaling molecules. When osseous healing exhausts the localized supply of cells and signaling molecules, renewal is contingent upon vascularity and operational activity of endogenous cells during the early phase of bone repair. Therefore the local blood supply performs a critical role in determining the success of bone healing. Nicotine has been reported to have effects directly on the small blood vessels in producing vasoconstriction, systemic venoconstriciton, and increasing coronary vascular resistance. (21-23) The intense vasoconstriction that nicotine exerts on the microvasculatures inhibits the angioblastic response during revascularization in the healing area and limits the recruitment of the related factors such as cytokines. These may explain the

adverse effects of nicotine on bone healing and has yet to be confirmed. Distraction osteogenesis provides an excellent model to explore the hard evidence of the effect of nicotine on bone regeneration.

Bone is formed mainly through two pathways: endochondral ossification in long bone development and repair, and intramembranous ossification in cranio-facial bone formation. The distraction regenerate in craniofacial distraction osteogenesis mineralizes predominantly via intramembranous ossification without a cartilage intermediate. Endochondral bone formation is considered unusual but could be observed occasionally during the early stage at the periphery of the distraction gap, where the callus outgrows its blood supply. Cartilage provides a suitable material that is less demanding on oxygen, which bridges the gap temporarily until the blood supply catches up after one to two weeks of consolidation. (24) Any reasons caused ischemia and low oxygen tension in the distraction regenerate may change the predominant bone formation pathway to endochondral ossification. The present study provides direct evidence that nicotine exposure can change the bone regeneration pathway. Obvious chondrocytes surrounded by a mineralised matrix were only noted in the high dose nicotine group, which indicated a decreased oxygen tension in the distraction regenerate when compared with the control and low dose nicotine groups. This is consistent with the previous reports that nicotine may cause vasoconstriction which finally result in delayed healing and proper mineralization.

Patients with heavy smoking habit, radiotherapy history, diabetes or long term steroid therapy demonstrates the similar compromised biological environment of the wound sites, such as vasoconstriction, tissue ischemia, compromised activity and recruitment ability of local cells and signaling molecules. These biological attentions of wound healing have important therapeutic and prophylactic indication to patients. The present study provides not only the medical evidence of the relationship between plasma nicotine concentration and the adverse impact of nicotine on bone regeneration, but also a reliable and convenient in vivo platform to further study the biological mechanisms and therapeutic options of the bone reconstructive surgery on compromised hosts.

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Abbreviations: HPLC: high-pressure liquid chromatography, micro-CT: Micro-computed Tomography, BV/TV: bone volume/tissue volume, EDTA: ethylenediaminetetraacetic acid

Key Words: Nicotine, Bone Healing, Distraction Osteogenesis, Rabbit

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