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Changes in blood perfusion and bone healing induced by nicotine during distraction osteogenesis

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ABSTRACT

Nicotine is the main chemical in cigarettes responsible for the tobacco's pathological effects. The influence of nicotine on bone healing remains controversial. Distraction osteogenesis provides an ideal model to study bone healing and regeneration. The present study aims to evaluate the effects of nicotine on blood perfusion, angiogenesis and bone formation using a rabbit model of mandibular lengthening. Twenty adult New Zealand white rabbits were randomly assigned to the control group and nicotine group. The total nicotine or placebo exposure time for all animals was 7 weeks. After 2- or 4-week of consolidation following osteotomy, 3-day of latency and 11-day of active distraction, the animals were sacrificed and the mandibles were harvested. Blood perfusion and vascularization were evaluated by Laser Doppler monitoring and Collagen IV immunohistochemistry staining respectively. Bone formation was assessed by radiological, histological and immunohistochemical examination. Results showed that nicotine exposure increased microvessel density, whereas inhibited blood flow and bone formation. The expression of bone morphogenetic protein (BMP)-2 in osteoblasts was also decreased. Frequent appearance of cartilage islands suggested ischemia and low oxygen tension in the distraction regenerate. We concluded that nicotine compromises bone regeneration possibly by causing ischemia and directly inhibitory effect on osteoblastic cells. Nicotine exposure enhances angiogenesis but cannot compensate for the adverse effect of vasoconstriction.

Introduction

Since the first clinical report of applying distraction osteogenesis to lengthen human mandibles [1], there is growing interest in surgeons on using this surgical method for the treatment of craniofacial deformities. Moreover, distraction osteogenesis provides an ideal model to study tissue healing and regeneration. It shares many features with embryonic growth, neonatal long bone development, and bone fracture healing [2,3]. The mechanical stimulation generated by controlled traction induces a cascade biological response of bone regeneration. The molecular signaling during distraction osteogenesis is amplified and prolonged as long as the mechanical traction is in progress.

Nicotine is the main chemical component responsible for tobacco addition [4]. It was reported to be of the highest importance among the potentially toxic substances in tobacco products [5,6], but its influence on bone healing remains controversial. Some studies indicated that nicotine has an adverse effect on bone healing and regeneration [5,7–9], while some other reports demonstrated no significant impact [10–12]. The controversial results were likely due to the variable dosages and methods of nicotine administration and the different wound healing models used to study the influence of nicotine. 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. Glowacki et al. validated a model of impaired distraction osteogenesis of the rat mandible with time release nicotine [13]. We have recently developed a rabbit model of mandibular lengthening to assess the effect of nicotine on bone regeneration using 60-day time release nicotine pellets [14]. Compared to the other nicotine delivery methods, surgical implantation of the time release nicotine pellets in the subcutaneous tissue can provide a reliable and convenient way to achieve a stable blood nicotine level for a long period [14]. Nicotine demonstrated 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. At a high dose nicotine exposure, the bone regeneration in distraction osteogenesis was significantly compromised [14]. This reliable rabbit model enables further research of the nicotine effect on bone healing mechanism.

The present study was designed to investigate the effect of nicotine on blood perfusion, angiogenesis and bone regeneration using a rabbit model of mandibular distraction osteogenesis.

Materials and methods

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

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University of Hong Kong. The animal experiment was approved by the Committee of the Use of Live Animals for Teaching and Research.

Nicotine implantation

Twenty adult New Zealand white rabbits (9-month old, 3.6–4.0 kg) were randomly assigned to the control group and nicotine group, with ten in each group. 1.5 g 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. The total nicotine exposure time for all the animals was 7 weeks (Fig. 1).

Osteotomy and distraction procedures

After nicotine implantation, a standard procedure of mandibular body osteotomy and distraction was performed [15]. Briefly, the animals were given a pre-operative 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 on one side of the mandibular body. A straight body osteotomy cut was made immediately anterior to the first premolar root. 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 2 weeks. For pain relief, buprenorphine (0.03 mg/kg) was administered subcutaneously twice daily for 10 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 3 days of latency period, distraction was activated at 0.9 mm once daily for 11 days. Five animals in each group were sacrificed after 2 and 4 weeks of consolidation. The mandibular samples were harvested for laboratory analysis and all the measurements were conducted blindly.

Laser Doppler monitoring

Blood flow in the distraction regenerate was measured using a laser Doppler perfusion monitor (LDPM, PeriFlux System 4001, Perimed, Stockholm, Sweden) before sacrificing the animals. Under anaesthesia, the skin and muscle were incised and elevated to expose the distraction regenerate. The laser probe (No.307) with PH 07-4 Miniholder was placed at 6 sites anterior and posterior to the distraction regenerate (Fig. 2). The LDPM uses the principle that light reflected from an object undergoes a frequency shift, and this energy shift in the light is proportional to the flow (concentration and velocity) of red blood cells moving past the probe. Perfusion was recorded in arbitrary perfusion units (PU) and the means represented the blood flow in anterior and posterior margins of the distraction regenerate.

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.

Micro-computed tomography (micro-CT)

After plain radiographic examination, the distracted tissue regenerate and a 3–5 mm section of the neighboring normal bone in the distracted mandible were harvested. The specimens were subjected to examination by a micro-CT machine μCT20 (Scano Medical AG, Bassersdorf, Switzerland) using a previously described protocol [15]. Briefly, each harvested specimen was placed into a 17-mm-diameter sample holder with the sagittal plane vertical to the X-ray tube and scanned with a slice increment of 100-μm. 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 a ROI on each section was calculated individually and a mean value of BV/TV for the total regenerate was obtained.

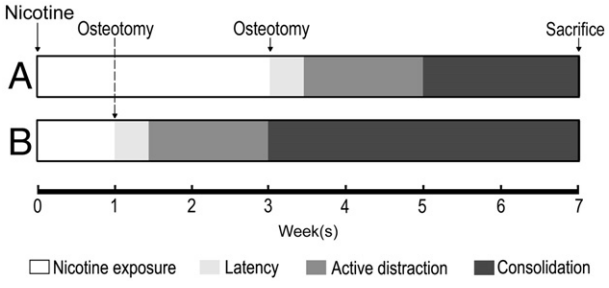

Fig. 1. The time line of nicotine exposure and distraction osteogenesis. A: rabbits sacrificed at week 2 of consolidation; B: rabbits sacrificed at week 4 of consolidation; Nicotine exposure: 7 weeks; latency period: 3 days; active distraction: 11 days.

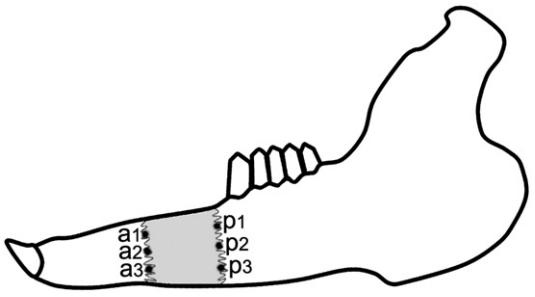

Fig. 2. The local blood perfusion is measured at three anterior sites (a1, a2, and a3) and three posterior sites (p1, p2, and p3) at the margin of the distraction regenerate.

Histology

After the micro-CT examination, the samples were decalcified in a solution of 14.5% ethylenediaminetetraacetic acid buffer (pH 7.2). The decalcified specimens were processed and embedded in paraffin wax. Axial sections of 5 μm in thickness were cut with a microtome and stained with haematoxylin and eosin for light microscopic examination.

Immunohistochemical staining

The sections were incubated with primary goat antibodies against Type IV collagen (Col IV, Southern Biotech, Birmingham, AB, USA) and BMP-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C. These antibodies have been confirmed to recognize the specific signals of rabbit BMPs and Col IV in previous studies [16,17]. For negative controls, the primary antibody was omitted. A goat ABC staining system kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for biotinylated second antibodies and horseradish peroxidase enzyme–avidin conjugate incubation according to the manufacturer's instructions. The sections were counterstained with haematoxylin.

To evaluate the microvessel density (MVD), microvessel number/(microvessel area+residual stromal area), the sections staining with Col IV were assayed by a standard technique described by Tao et al [18] using a computer-assisted image analyzing system (Leica DC 300 V 2.0, Leica, Wetzlar, Germany) with a morphometric software (Qwin V 2.4, Leica, Cambridge, UK). Col IV is a component of the basal lamina of vessels. Its staining allows proper identification in the case of immunohistochemical analysis of blood vessels. Any single brown-stained cell or cluster of endothelial cells that was clearly separated from adjacent microvessels, hisocytes, and other connective tissue element was considered a vessel [18]. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. Slides were screened at ×1.6 magnifications by light microscopy. 4 corner areas and 1 center area in distraction regenerate were identified. Then, vessels were counted in the 5 areas at ×20 magnifications (324390.41 μm^2 per area).

The cells expressing BMP-2 in the distraction regenerate were counted using a standard cell-counting technique described in our previous study [16]. The expression intensity of BMP-2 was defined as:−, no positive staining;+, less than one-third of cells positive;++, one-third to two-thirds of cells positive;+++, more than two-thirds of cells positive.

Statistical test

The values between the two groups were compared by two sample *t*-test with version 11.0 of Statistical Package of Social Sciences software (SPSS Inc., Chicago, USA). A statistical result of 0.05 was considered as significant.

Results

Clinical examination

All twenty rabbits completed the experimental process uneventfully. All rabbits showed mild weight loss after the operation and

Table 1
Mean (SD) of blood perfusion (PU) in the rabbit mandibular distraction regenerates (n=20)

Group	Anterior		Posterior	
	Week 2	Week 4	Week 2	Week 4
Nicotine	52.1 (8.24)	76.6 (5.02)	98.7 (8.65)	121.5 (7.67)
Control	69.4 (6.26)	91.1 (6.38)	116.2 (7.65)	139.8 (8.29)
P	0.0057	0.0213	0.0039	0.0067