

Applicability of histomorphometry analysis for evaluating alveolar bone regeneration after gene transfer

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It can be difficult for researchers and clinicians to evaluate bone tissues after some regenerative bone treatments because bone is always undergoing the process of remodeling. Moreover, hard tissues such as bone or teeth are typically very difficult to handle to prepare fresh and proper materials for clinical use. Therefore, regenerative bone tissues must be evaluated by adequate methods. In our previous studies, we developed a bone regeneration system using a combination of a non-viral bone morphogenetic

protein gene-expression plasmid vector and *in vivo* electroporation. Using a model of ectopic bone formation in the skeletal muscles of rats made it easy to evaluate the bone-inductive potential of our developed system. In this study, we used bone morphometry analysis to evaluate the alveolar bone with our gene-transfer system. Upon labeling alveolar bone with calcein and tetracycline in rats and analyzing double-staining, we concluded that histomorphometry analysis is an efficient method for evaluating alveolar bone regeneration induced by a combination of non-viral plasmid vector and *in vivo* electroporation.

Key Words: Alveolar bone; Histomorphometry analysis; Bone labeling; Gene therapy

Bone regeneration therapy is pivotal for many patients with bone defects caused by trauma, tumor or malformation (1), and alveolar bone regeneration therapy is important for the maintenance of teeth (2). Therefore, many researchers are endeavoring to identify efficient and safe therapies for bone regeneration (3). In these studies, it is sometimes difficult to evaluate the bone regenerated by their developed methods because bone is always undergoing the process of remodeling (4). Moreover, hard tissues such as bone or teeth are difficult to handle to yield fresh and proper materials (5). For these reasons, we typically used ectopic bone formation models in the skeletal muscles of rats to evaluate bone regeneration methods (6,7). However, it is also necessary to evaluate regenerated bone within target bone defect areas for clinical applications.

In previous studies, we developed a gene-transfer system for bone regeneration therapy by combining a non-viral gene expression plasmid vector and *in vivo* electroporation (8,9). The ultimate goal is to apply this method for alveolar bone regeneration (10). Therefore, we require an adequate evaluation method for regenerated alveolar bone after gene therapy treatment. In this study, we used histomorphometry analysis to evaluate alveolar bone regeneration induced by our gene-transfer system.

MATERIALS AND METHODS

Gene transfer

Nine-week-old male Wistar rats (n=3 per group) were anesthetized via intraperitoneal injection of pentobarbital sodium (5.0 mg/100 g of body weight). The lacZ non-viral gene expression vector detailed in our previous study (11) was diluted to 0.5 µg/µL in phosphate-buffered saline, and a total volume of 50 µL was injected into the palatal region of periodontal tissues in the first molar of the right maxilla using a syringe with 31-gauge needle. Electroporation was then performed immediately, with 32 electrical pulses at 50 V for 50 msec (12).

Double-staining of bone

Rats (n=3 per group) were intraperitoneally injected with calcein (10 mg/kg) on the day of initiation of gene transfer. Three days later, tetracycline hydrochloride (30 mg/kg) was intraperitoneally injected. Six days after gene transfer, rats were again injected with calcein. Seven days after gene transfer, rats were sacrificed with an overdose of pentobarbital sodium. The schedule of bone labeling is described in (Figure 1). The maxillary regions of rats were dissected and fixed with 70% ethanol for 8 days, followed by staining with Villanueva bone stain for 10 days, dehydration with increasing concentrations of ethanol, and embedding in methyl methacrylate without

decalcification. After polymerization, frontal 10-µm sections were obtained from the mesiolingual center of the upper first molars and second molars. Sections were observed by fluorescence microscopy under UV irradiation for tetracycline (364 nm) and calcein (477 nm) labeling. The width of areas labeled with calcein and tetracycline was measured vertically at 10 points within the region in which gene transfer had been performed, using a Histometry RT Camera (System Supply, Tokyo, Japan). Statistical analyses were performed by unpaired t test.

RESULTS

Villanueva bone staining

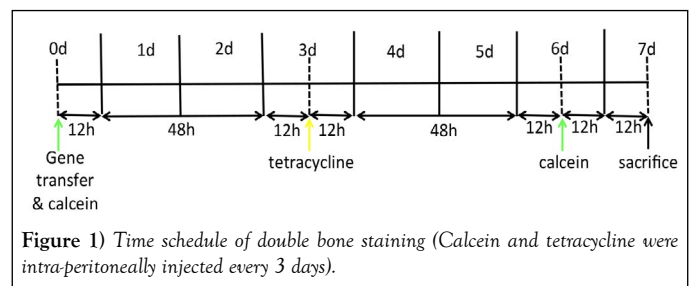
Villanueva bone staining was visualized as follows: transparent green to jade green or homogeneous red represented osteoid, red represented low-density bone, greenish-blue to dark purple represented nuclei of osteoblasts or osteoblasts, and green or light green represented cytoplasm. After gene transfer, no significant differences in osteoid or low-density bone were observed between control and gene-transfer sites (Figure 2A).

Bone labeling and MAR

We found three labels in the alveolar bone of both control and gene-transfer sites (Figure 2B). Moreover, we did not observe significant differences in the width of each label between control and gene-transfer sites. Results of MAR from 0-3 days and 3-6 days after gene transfer were not significantly different from the control sites (Figure 3).

DISCUSSION

After transferring a lacZ non-viral vector into the periodontal tissues of rats with electroporation, we revealed that lacZ gene transfer with electroporation did not influence alveolar bone formation and remodeling



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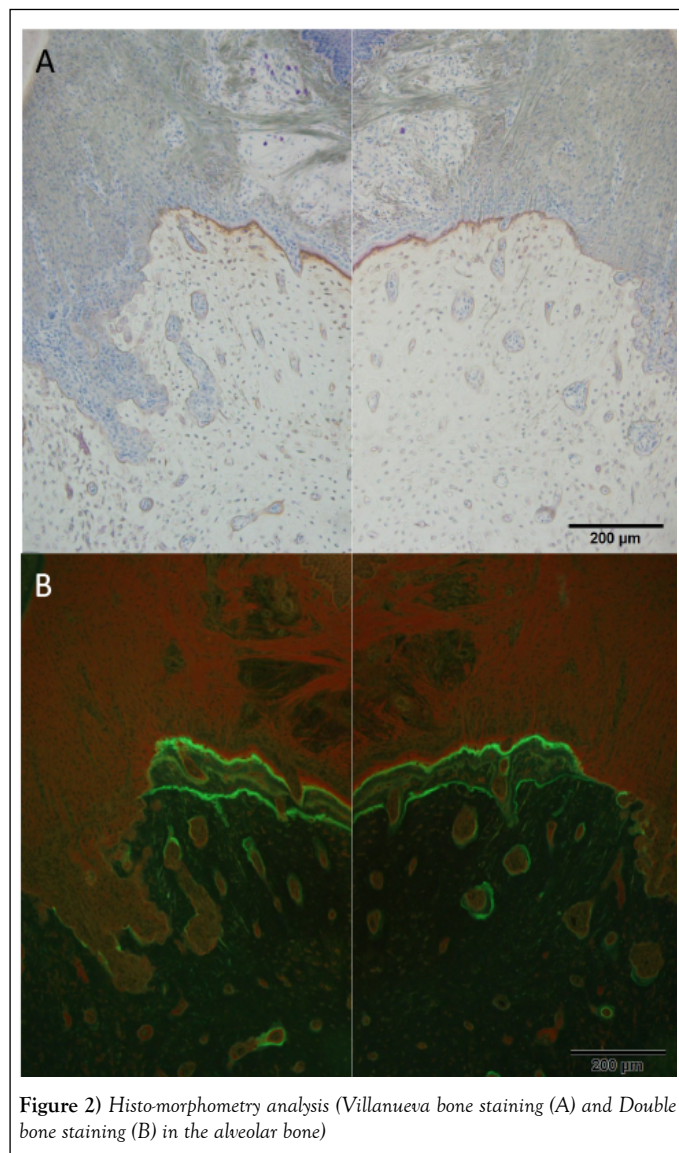


Figure 2) Histo-morphometry analysis (Villanueva bone staining (A) and Double bone staining (B) in the alveolar bone)

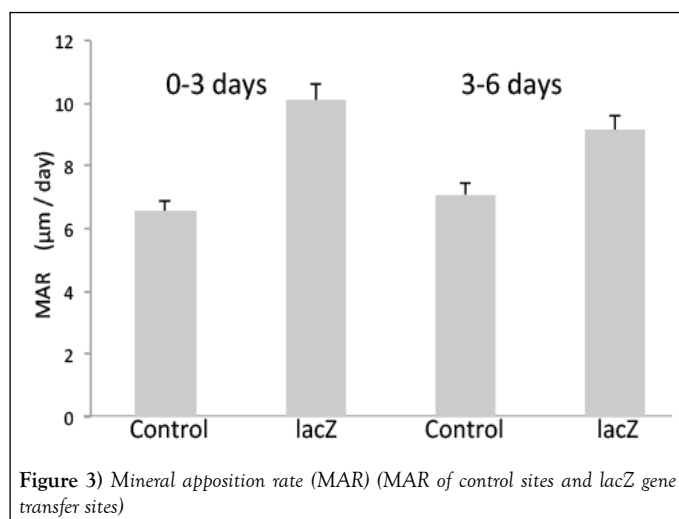


Figure 3) Mineral apposition rate (MAR) (MAR of control sites and lacZ gene transfer sites)

using histomorphometry analyses. In our developed gene-transfer system, electrical pulses are needed to induce the efficiency of gene transfection (8). However, some reports have described the influence of electronic pulses on bone formation (13,14). In this study, histomorphometry analyses of alveolar bone after lacZ gene transfer with electroporation revealed that electrical stimulation did not influence alveolar bone regeneration.

Preparing histomorphometry samples is sometimes difficult and requires a lot of time because it is not possible to accelerate decalcification (5).

However, histomorphometry analyses can reveal the time-variant continuous conditions of bones (15). In contrast, histological staining of sections with hematoxylin and eosin demonstrates only a snapshot or the fragmental changes of bone. This is an important distinction, as bone is always remodeling and changing (15).

CONCLUSION

We concluded that histomorphometry analysis of alveolar bone with gene-transfer treatment is effective for evaluating regenerated bone. In the future, we plan to analyze alveolar bone treated with electroporation transfer of osteogenic cytokine genes, such as bone morphogenetic protein, by histomorphometry analysis.

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