It has been reported that occlusal forces promote periodontal healing of transplanted teeth and prevent dentoalveolar ankylosis, although its mechanism is still unclear. Nitric oxide (NO) produced by NO synthase (NOS) is considered to be an important factor which is involved in wound healing, and it increases with mechanical stimuli. The objective of this study was to examine the relationship among occlusal stimuli, inducible NOS (iNOS) and PDL healing of transplanted teeth. Five-week-old Sprague-Dawley male rats were used for this study. The right maxillary first molars of rats were replanted and animals were divided into occluded and non-occluded groups. Histologic observations were carried out after one and two weeks. After two weeks, the non-occluded group had clearly detectable ankylosis and obvious PDL stricture. On the other hand, the occluded group showed an enlarged and thickened PDL without ankylosis. The number of iNOS positive cells in the occluded group, samples significantly increased in comparison to that of the non-occluded group. These results suggest that occlusal stimuli enhanced the production of NO in the PDL healing process of transplanted teeth and a favorable result could be obtained.

Key words: transplantation of teeth, mechanical stimuli, periodontal healing, nitric oxide

Introduction

The history of autotransplantation of human teeth can be traced back to ancient times. With the progress of modern medicine, the success rate of autotransplantation of human teeth has been greatly augmented with its survival rate up to 90% in recent years. Clinically, autotransplantation has been widely performed to substitute missing teeth, and it has the advantage of functional adaptation and preservation of the alveolar ridge. However, about 23% of transplanted teeth have unfavorable results, most of them caused by dentoalveolar ankylosis.

Periodontal ligaments (PDL) are filled with abundant blood vessels, collagen fiber, and extracellular substances, and have a high ability to regenerate and to remodel themselves. Some studies reported that in order to maintain the normal function of PDL, proper mechanical stimuli, such as occlusal forces, are necessary. Loss of normal occlusion causes atrophic changes in the PDL, including narrowing of the
PDL space, disorientation of collagen fibers and blood vessel constriction. Recently it has been reported that occlusal forces could promote periodontal healing of transplanted teeth and prevent dentoalveolar ankylosis. However, the relationship between mechanical stimuli and the healing process of the PDL in transplanted teeth is still unclear.

In normal PDL, mechanical stimuli can enhance several local factors including nitric oxide (NO). NO, a biologically active molecule that has various functions, is synthesized from L-arginine in a process catalyzed by nitric oxide synthase (NOS). NOS is produced by many types of cells and is classified into three types: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS). Among them, iNOS and eNOS have been identified in PDL. The production of iNOS is mainly in fibroblasts and macrophages, and eNOS is only noted in blood vessels. Both of them are involved in the recovery process of hypofunctional condition to normal condition. In addition, NO plays many important roles in wound healing, such as regulating proliferation and differentiation of several cell types, modulating collagen formation and angiogenesis. Moreover, the blockade of NO synthesis was reported to impair wound healing. Although little is available on the relationship between NO and the PDL healing process of transplanted teeth, NO is considered to be a key mediator in PDL healing. We focused on iNOS in the present study, and its objective is to verify the hypothesis that NO is involved in the healing process of the PDL of transplanted teeth, on which occlusal force was applied to obtain better PDL healing and to prevent dentoalveolar ankylosis.

Materials and Methods

Twenty-four 5-week Sprague-Dawley (S.D.) male rats, mean weight of 150±15 g, were used in this study. All procedures followed the guidelines of the Tokyo Medical and Dental University for Animal Research. The experimental protocols had the approval of the Animal Welfare Committee.

In order to examine the role of NO in PDL with or without occlusal stimuli, we divided the animals into an occluded and a non-occluded group. In the non-occluded group, an anterior bite plate and a metal cap were attached to the maxillary and mandibular incisors, respectively, using a light-curing composite resin (Clearfill Liner Bond II, Kuraray, Okayama, Japan) in order to disocclude molar regions (Figure 1A). All experiments were performed under anesthesia by intraperitoneal injection with ketamine hydrochloride (KETARAL 50; Sankyo, Tokyo, Japan) containing 20% xylazine hydrochloride (Celactal 2% injections; BAYER-Japan, Tokyo, Japan). The right maxillary first molars of all animals were replanted according to the method described by Kvinnsland et al. The teeth were extracted using tissue forceps, rotated once anteriorly so that all roots came out of the socket while leaving a part of the attached mesial gingiva intact (Figure 1B), and then immediately repositioned. No postoperative splinting was used.

Animals were perfused intracardially through the left ventricle with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4 under anesthesia at 1 or 2 weeks after replantation. The maxillary specimen was removed en bloc and immersed in the same fixative at 4°C for 2 hours, and decalcified in 4% EDTA solution at 4°C for 6-8 weeks. Decalcified tissues were immersed overnight in 30% sucrose solution at 4°C and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Europe, Zoeterwoude, Netherlands). Twenty μm thick serial sections were cut horizontally using a cryostat (Leica CM3000, Nussloch, Germany), and mounted on poly-L-lysine coated glass slides (Matsunami, Osaka, Japan). The observation site was determined at the level of 500-600 μm from the furcation of the disto-palatal root of the maxillary first molars (Figure 2A). This site was chosen because it is approximately at the midsection of the root and the cross section of the disto-palatal root is...
round, making comparisons of PDL conditions easier. The disto-palatal root was chosen because our preliminary experiments had shown it to have the least variations in morphology, and this root could be moved out completely from the socket during the operation of replantation.

**Immunohistochemistry**

The immunostaining protocol for iNOS was identical to that of Watarai et al., and iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) was used.\(^20\) The prepared sections were washed in 0.01M phosphate buffered saline (PBS) for 15 minutes, and treated with 0.3% H\(_2\)O\(_2\) in absolute methanol for 30 minutes in order to inactivate endogenous peroxidase. After preincubating in 2% normal horse serum (Vector, Burlingame, CA, USA) in 0.01M PBS for 30 minutes, sections were incubated with iNOS rat monoclonal antibody, which were diluted at 1:50 with 2% normal horse serum, for 30 minutes at 37°C humid atmosphere; this was followed by two consecutive incubations with biotinylated anti-mouse IgG (1:200) and avidin-biotin-peroxidase (ABC) complex, respectively (ABC-PO kit, Vector, Burlingame, CA, USA), for 30 minutes. All incubations were followed by 15 minutes of washing with PBS. Immunoreactivity was visualized using 0.02% 3,3-diaminobenzidine tetrahydrochloride (Dojin Chemical, Kumamoto, Japan) and 0.01% H\(_2\)O\(_2\) in 0.05 M Tris-HCl buffer (pH 7.6). The immunostained sections were finally mounted with 70% glycerin.

**Quantitative analysis**

Measurements were performed in a square area of interest (200 \(\mu m \times 200 \mu m\), Figure 2B). We drew the buccopalatal line that passed through the central point of the root (which is line \(\alpha\)). Point P was determined as the intersection of the line \(\alpha\) and the border-line between the palatal PDL and alveolar bone. We drew the line \(\beta\) that passed through point P and was perpendicular to the line \(\alpha\), and then a square was made as in Figure 2B. Then the number of iNOS-immunopositive cells in the area was counted.

The immunostained specimens were observed and photographed by a light microscope (Microphoto-FXA, Nikon, Tokyo, Japan) equipped with a digital camera (DXm1200, Nikon, Tokyo, Japan), and stored in 24-bit true-color TIFF format. Measurement was performed three times in the representative section obtained from the six samples of each group by means of image analysis software (Image-Pro, Media Cybernetics, Silver Spring, MD, USA)\(^20\). Analogic microscopic images were converted into digital images, and the image analysis program was used to establish a threshold for the measurement of immunopositive cells. The threshold that defined the cell margin was established as the method described by Watarai et al.\(^20\), then the number of iNOS-immunopositive cells was counted manually by three investigators. Six rats in each group were used to count iNOS-immunopositive cells. Each section was counted on three different days and 5 consecutive sections per animal were counted to correct differences in observation. The number of iNOS-immunopositive cells were analyzed by ANOVA followed by Scheffe’s *post hoc* test (\(p<0.05\)) using Stat View 5.0J software (SAS Institute, Cary, NC, USA).

**Results**

No obvious morphological difference was detected in both occluded and non-occluded groups at first week (Figure 3A,B). After two weeks, ankylosis was clearly observed on the disto-palatal side (Figure 3C) in the non-occluded group. On the other hand, in the occluded group after two weeks, ankylosis was not found, the width of the PDL widened and root resorption was noted (Figure 3D).

Inducible NOS was identifiable in blood vessels, fibroblasts and cells of mononuclear phagocyte lineage in normal PDL, and it greatly decreases in non-
occluded PDL (data not shown). In the non-occluded group both after 1 week and after 2 weeks, the number of iNOS-immunopositive cells decreased significantly (Figure 4A,C; Figure 5). In contrast, in the occluded group both after 1 week and after 2 weeks, the number of iNOS-immunopositive cells significantly increased. (Figure 4B,D; Figure 5).

**Discussion**

In our study, iNOS was observed in the occluded group both after one week and after two weeks. From quantitative analysis, the number of iNOS-immunopositive cells greatly decreased in the non-occluded group. NO plays many important roles in immune function and the wound healing process, and impaired wound healing is accompanied by decreased wound NO synthesis. In addition, wound NO synthesis inhibition was reported to cause poor result of wound healing, and the blockade of iNOS impairs collagen synthesis, reduces proliferation of fibroblast, reduces vascular endothelial growth factor (VEGF) expression in wound healing.
process, and impairs regenerative outcome in peripheral nerve. Therefore, an insufficiency of iNOS may lead to a poor result of the PDL healing in transplanted teeth and cause dentoalveolar ankylosis. These results suggest that NO production could be enhanced by occlusal stimuli in the PDL of transplanted teeth, and the existence of NO is necessary for the repair of injured PDL fibers, angiogenesis and even for nerve regeneration.

On the other hand, it has been reported that iNOS induces osteoblasts apoptosis and depresses bone formation, and the inhibition of iNOS was reported to reduce the number of osteoclasts in PDL. Consequently, with the existence of iNOS, PDL width could be maintained and this may keep the root surface away from direct contact with alveolar bone and avoid the occurrence of dentoalveolar ankylosis. Although little is available on the relation between iNOS and the process of calcification, iNOS can be regarded as a factor that takes part in the prevention of ankylosis.

In summary, complete PDL repair of transplanted teeth is considered to be the key factor which may prevent poor results such as dentoalveolar ankylosis. Enhanced iNOS is important for PDL repairing and maintaining PDL width, and decreases the possibility of dentoalveolar ankylosis. We regard dentoalveolar ankylosis as an unfavorable condition of teeth.

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