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ORIGINAL ARTICLE

Periodontal Healing by Implantation of Collagen Hydrogel-sponge Composite in One-wall Infrabony Defects in Beagle Dogs

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SYNOPSIS

The purpose of this study was to evaluate the effect of implanted collagen hydrogel-sponge composite on periodontal wound healing. One-wall infrabony defects (depth: 5 mm; width: 3 mm) were surgically created in three beagle dogs. The exposed root surface was planed and demineralized with EDTA. In the experimental group, the defects were filled with collagen hydrogel-sponge composite. Conversely, no collagen hydrogel-sponge composite was applied to defects in the control group. Histomorphometric parameters were evaluated four weeks after surgery. In the experimental group, regeneration of alveolar bone and cementum was frequently observed. Periodontal ligament tissue was reestablished between the alveolar bone and cementum. New bone height, new bone area, new cementum and new periodontal ligament in the experimental group were significantly greater than those in the control group ($p < 0.05$). These findings suggest that implanted collagen hydrogel-sponge composite facilitates periodontal wound healing in one-wall infrabony defects in beagle dogs.

Key words: collagen hydrogel sponge composite, scaffold, periodontal wound healing

INTRODUCTION

A biological scaffold plays an important role in differentiation, angiogenesis, and maintaining space for cell repopulation and extracellular matrix reconstruction in regenerative therapy¹. Various scaffolds, such as collagen², gelatin³, calcium phosphate⁴ and synthetic polymer⁵, have been used in previous studies. In particular, type I collagen has been widely used as a tissue graft^{2, 6-8}, be-

cause it is easy to upregulate its denaturation, cross-linking and concentration pattern for a scaffold⁹. The regenerative scaffold requires rapid replacement following early cell migration and tissue reconstruction at the wound site. Shimoji *et al.*¹⁰ demonstrated that implanting sponge-type collagen into the rat femur maintained tissue growth space and stimulated bone augmentation, and concluded that collagen

sponge acted as a stable scaffold for osteogenic cells. However, few cells were observed in the inner part of the collagen sponge, suggesting that infiltration of tissue-forming cells into the sponge, especially into the central region, may be limited.

Recently, a collagen hydrogel treated by alternative cross-linking with the ascorbate-copper ion system was developed¹¹. Matsui *et al.*¹² reported that the collagen hydrogel possessed high tissue-compatibility and high replacing ability following in vivo application, and enhanced wound healing in rat skin defects. Furthermore, Ishizuka *et al.*¹³ and Miyaji *et al.*¹⁴ reported that numerous cells infiltrated into the inner side of the collagen hydrogel following application to periodontal dehiscence-type defects in beagle dogs, stimulating alveolar bone and cementum formation. Therefore, the collagen hydrogel acts as a regenerative scaffold for periodontal cells. However, in the case of advanced periodontal defects, such as one-wall or horizontal defects, it is difficult to maintain the collagen hydrogel in the defect due to the hydrogel's high fluidity and low mechanical stiffness.

To enhance cell proliferation and migration, we prepared a collagen hydrogel-sponge composite in which the collagen hydrogel was injected to fill spaces in the collagen sponge. Spongiform collagen seems to acquire the cell proliferative activity of the collagen hydrogel. Tokunaga *et al.*¹⁵ reported that a collagen hydrogel-sponge composite stimulated bone augmentation following implantation in rat cranial bone. Rapid cell infiltration in the material and replacement by regenerative tissue with low cytotoxicity was also observed. Thus, we speculated that implantation of collagen hydrogel-sponge composite would induce reconstruction of periodontal tissue in large defects, because periodontal cell growth in the defects

was stimulated. The purpose of the present study was to evaluate whether implanted collagen hydrogel-sponge composite facilitated periodontal wound healing in one-wall infrabony defects.

MATERIALS AND METHODS

Preparation of collagen hydrogel

Collagen hydrogel was prepared from atelocollagen powder (Atelocollagen powder T, Lot No. 030310, Koken, Tokyo, Japan) by adding 5 mM HCl, 1 mM L(+)-ascorbic acid and 0.1 mM CuCl₂ and adjusted to a final concentration of 1.5%.

Preparation of collagen sponge

Collagen sponge was provided by Olympus Terumo Biomaterials (Tokyo, Japan). Atelocollagen in a dilute HCl solution (0.3%; pH 3) was neutralized by adding concentrated phosphate buffer in NaCl to a final concentration of 0.1% collagen, 30 mM Na₂HPO₄ and 0.1 M NaCl. This collagen solution was incubated at 37°C for 4 hours. The resulting fibrous precipitate is herein referred to as fibrillar collagen (FC). Heat-denatured collagen (HAC) was prepared from atelocollagen in a dilute HCl solution by heating at 60°C for 30 minutes. A composite of FC (0.3%) and HAC (1%) was prepared by mixing the two at a ratio of 9:1 (w/w), respectively. This composite material was adjusted to a final concentration of 4% and made in the form of a sponge by lyophilization at -30°C. This sponge was then dehydrothermally cross-linked at 110°C for 2 hours. Eighteen pieces of the resulting FC-HAC sponge (5 × 5 × 3 mm) were used in the present study.

Preparation of collagen hydrogel-sponge composite

Collagen hydrogel (100 µl) was injected into the FC-HAC sponge. The construct was immediately immersed in phosphate-buffered saline (PBS, pH 7.2) to

acquire viscoelasticity at neutral pH.

Animals

Three healthy female beagle dogs, 12-16 months old and weighing approximately 10 kg, were used in this experiment. The experimental protocol followed the institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No. 08-0255). Surgical procedures were performed under general anesthesia with medetomidine hydrochloride (0.1 ml/kg, Domitor, Nippon Zenyaku Kogyo, Koriyama, Japan) and butorphanol tartrate (0.1 ml/kg, Betorphanol, Meiji Seika, Tokyo, Japan), and under local anesthesia with lidocaine hydrochloride (2% with 1:80,000 epinephrine, Xylocaine, Dentsupply-sankin, Tokyo, Japan).

Surgical procedure

The mandibular first and third premolars were extracted before the experimental surgery, and the extraction sites were allowed to heal for eight weeks. Following reflection of the buccal and lingual muco-gingival flaps with partial thickness, periosteum was removed from alveolar bone. Eighteen one-wall infrabony defects (depth: 5 mm; width: 3 mm) were surgically created in the mesial and distal aspect of the mandibular second premolars, and the mesial aspect of the mandibular fourth premolars (Fig. 1-A). The root surface facing the defect was planed to remove cementum. Reference notches indicating the cemento-enamel junction and bottom of the defect were prepared on the root surfaces. Defects were then randomly assigned to the experimental and control groups. Subsequently, the denuded root surface was demineralized with 24% EDTA (pH 7.0) for 3 minutes and washed with saline. In the experimental group, the defect was filled with collagen hydrogel-sponge composite (Fig. 1-B)

and the flap was repositioned and securely sutured (Surgilon, Tyco Healthcare Japan, Tokyo, Japan). In the control group, the flap was closely sutured without implantation of collagen hydrogel-sponge composite. The animals received ampicillin sodium (300 mg/kg, Viccillin, Meiji Seika, Tokyo, Japan) daily for three days and a plaque control regimen with 0.5% chlorhexidine twice weekly for the entire duration of the experiment.

Histological procedure

The animals were euthanized using an overdose of sodium pentobarbital (0.5 ml/kg, Somnopentyl, Kyoritsu, Tokyo, Japan) following general anesthesia with medetomidine hydrochloride and butorphanol tartrate. Specimens were collected from the wound four weeks post-surgery. The tissue blocks, including teeth, bone and soft tissue, were fixed in 10% buffered formalin, decalcified in 10% formic-citric acid, and embedded along the mesial-distal plane in paraffin wax. Sections (6 µm thick) were serially prepared and stained with hematoxylin-eosin (HE) and Masson's trichrome stains.

Histomorphometric analysis

Three HE-stained sections were taken; one was approximately from the center of the root, and the other two were 180 µm from either side of the center. The following nine histomorphometric measurements were performed for each stained section using a software package (ImageJ 1.41, National Institute of Health, USA):

1. Defect height: distance between the apical notch and the cemento-enamel junction.
2. New bone height: distance between the apical notch and the coronal extension of the newly formed alveolar bone along the root surface.
3. New bone area: newly formed alveo-

lar bone in the defect area.

4. New cementum: distance between the apical notch and the coronal extension of the newly formed cementum-like tissue on the root surface.

5. New periodontal ligament: length of functional fibrous tissue between the newly formed cementum-like tissue and alveolar bone.

6. Ankylosis: length of ankylotic union of newly formed alveolar bone and the root surface.

7. Gingival connective tissue: distance between the apical extension of junctional epithelium and the coronal extension of alveolar bone or cementum.

8. Junctional epithelium: distance between the cemento-enamel junction and the apical extension of junctional epithelium.

9. Gingival recession: distance between the cemento-enamel junction and the gingival margin.

Statistical analysis

The means and standard deviations of each parameter were calculated for two groups. Differences between the groups were analyzed using Student's t-test. P-values < 0.05 were considered statistically significant. All statistical procedures were performed using SPSS (ver. 11.0, SPSS Japan, Tokyo, Japan).

RESULTS

Histological observations

Due to pulp tissue exposure during the creation of bone defect in the surgical procedure, one specimen was eliminated from this study. Otherwise, post-operative healing was uneventful in all dogs.

In the control group, newly formed connective tissue filled the defect in most areas. Gingival recession and downgrowth of junctional epithelium were frequently observed. Gingival recession was evident in four defect sites (4/8). Small amounts of bone and ce-

mentum had formed in the apical portion of the defect. No ankylosis or root resorption was observed. (Fig. 2)

In the experimental group, formation of new bone was observed in the defect (Fig. 3-A). Newly formed bone comprised osteocyte and trabecula lined by osteoblastic cells. In the coronal portion, the periodontal defect was filled with cell-rich connective tissue containing many blood vessels (Fig. 3-B). However, downgrowth of junctional epithelium was seen at the coronal portion of the root surface. Cementum-like tissue, continuous with the original cementum, was observed on the root dentin surface (Fig. 3-C). There was no resorption with osteoclastic cells on the root surface. Sharpey's fibers inserting into both the new cementum-like tissue and alveolar bone were seen, indicating that functionally oriented periodontal ligament tissue was reestablished (Figs. 3-C, 4-A, and 4-B). Little of the collagen hydrogel-sponge composite remained. Few inflammatory cells were seen around the residual collagen hydrogel-sponge composite (Fig. 3-D). Gingival recession was evident in one defect site (1/9).

Histomorphometric analysis

Mean defect height (mm) was 4.76 ± 0.19 and 4.83 ± 0.41 in the control and experimental groups, respectively, with no significant differences between the defects. Height of new bone (mm) was 0.81 ± 0.32 and 1.89 ± 0.52 , and the area of new bone (mm^2) was 1.64 ± 0.70 and 4.45 ± 1.05 in the control and experimental groups, respectively. Significant new bone formation was observed in the experimental group as compared to the control group. Significant formation of new cementum (mm) was also observed in the experimental group (1.81 ± 0.75) as compared with the control group (0.37 ± 0.26). New periodontal ligament (mm) measured

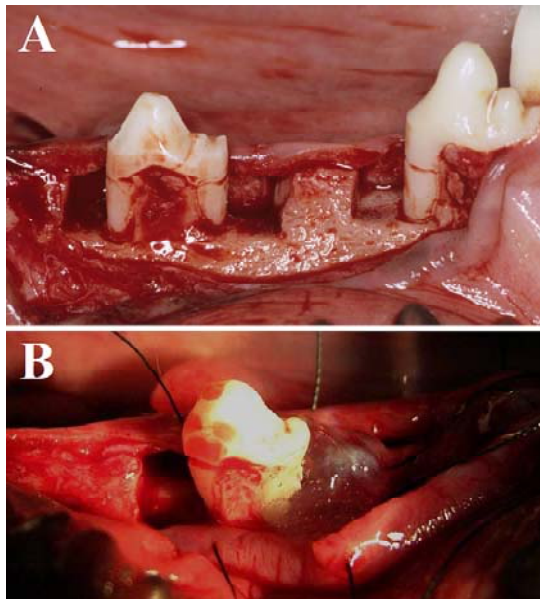


Figure 1
A) One-wall infrabony defects were surgically created. B) The defect was filled with collagen hydrogel-sponge composite.

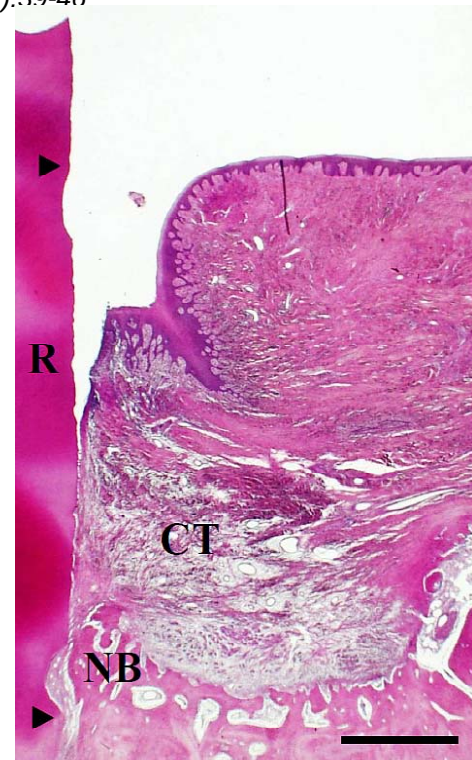


Figure 2
Histological findings in the control group. Newly formed connective tissue filled the defect in most areas. R, root; NB, new bone; CT, connective tissue; arrowhead, reference notches; HE staining; scale bars: 1 mm.

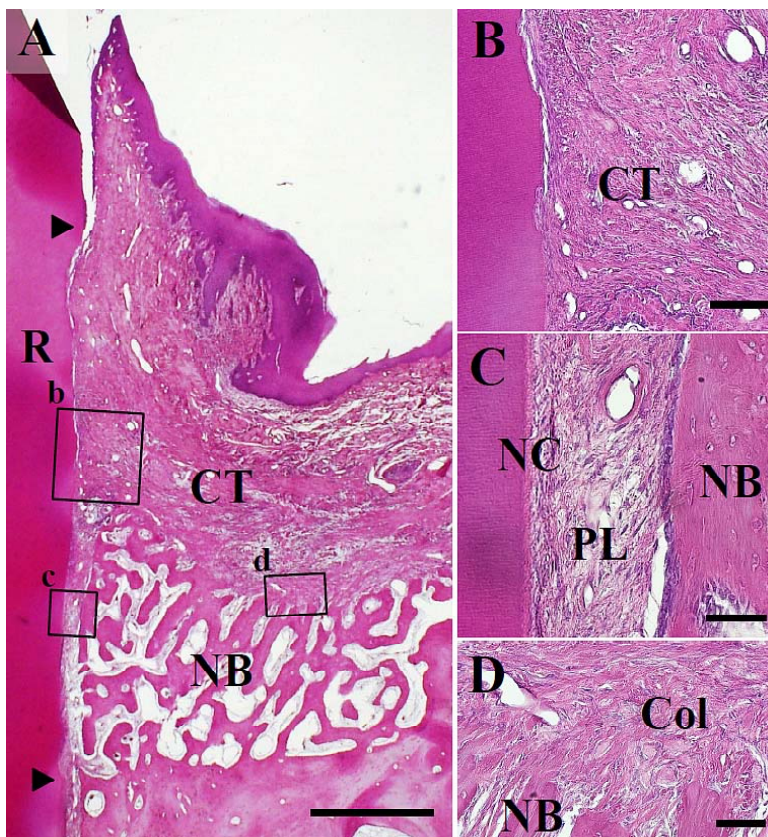


Figure 3
Histological findings in the experimental group. A) New bone was frequently formed in the defect. B) Higher magnification of the framed area (b) in A. Defect was filled with cell-rich connective tissue containing many blood vessels. C) Higher magnification of the framed area (c) in A. Cementum and periodontal ligament were observed on the root dentin surface. D) Higher magnification of the framed area (d) in A. Few inflammatory cells were seen around the residual collagen hydrogel-sponge composite. R, root; NB, new bone; CT, connective tissue; NC, new cementum; PL, periodontal ligament; Col, collagen hydrogel-sponge composite; arrowhead, reference notches; HE staining; scale bars: A = 1 mm; B = 100 µm; C = 50 µm; D = 50 µm.

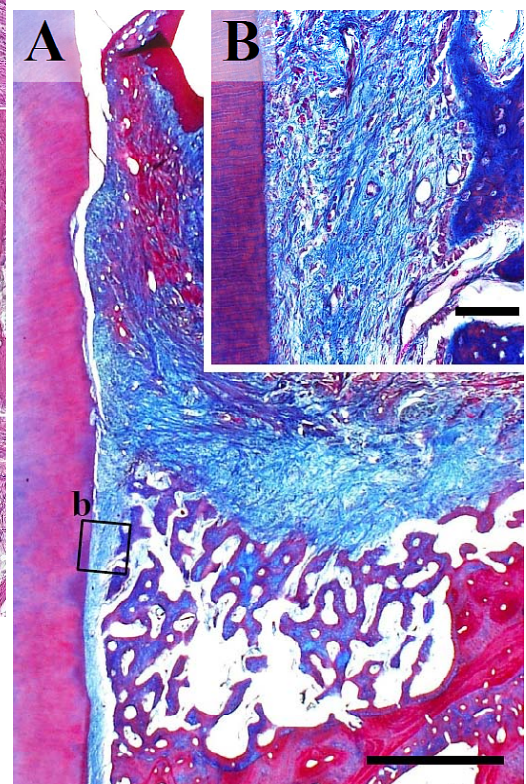


Figure 4
A) Histological findings in the experimental group. B) Higher magnification of the framed area (b) in A. Functionally oriented periodontal ligament tissue was reestablished. Masson's trichrome staining; scale bars: A = 1 mm; B = 50 µm.

1.36 $\pm$ 0.58 in the experimental group, which was significantly greater than the 0.34 $\pm$ 0.25 measured in the control group. Ankylosis slightly occurred in the experimental group (0.02 $\pm$ 0.07 mm). Gingival connective tissue (mm) was 2.97 $\pm$ 0.97 and 1.64 $\pm$ 0.98 in the control and experimental groups, respectively. Substantive attachment of gingival connective tissue to the root surface was seen in the control group compared to the experimental group. No significant differences in downgrowth of the junctional epithelium (0.90 $\pm$ 0.95 vs 0.93 $\pm$ 0.95 mm, respectively) or gingival recession (0.34 $\pm$ 0.52 vs 0.11 $\pm$ 0.33 mm, respectively) were seen between the control and experimental groups (Table 1).

DISCUSSION

The present study focused on periodontal wound healing following implantation of collagen hydrogel-sponge composite in one-wall infrabony defects in beagle dogs. In the experimental group, alveolar bone formation was stimulated by implants. Matsui *et al.*¹² and Miyaji *et al.*¹⁴ reported that a collagen hydrogel possessed high tissue-compatibility and replacing ability, and its implantation allowed early tissue formation in vivo. Furthermore, the collagen hydrogel-sponge composite stimulated cell infiltration and bone

augmentation in rat cranial bones¹⁵. We speculated that alveolar bone-derived cell attachment and population would be promoted by collagen hydrogel application. Miyamoto *et al.*¹⁶ reported that the expression of alkaline phosphatase, osteopontin, and bone sialoprotein (markers of osteoblastic phenotype), by bone marrow stromal cells were enhanced by cell culture using type I collagen gel. Mizuno *et al.*¹⁷ revealed in a mouse study that subcutaneous implantation of bone marrow stromal cells incubated in type I collagen gel caused ectopic bone formation. Many investigators have attempted to elucidate the process of collagen calcification in bone formation^{18, 19}. Therefore, it seemed likely that application of collagen hydrogel-sponge composite would stimulate osteoblastic differentiation in periodontal defects.

We also found that significant cementum and periodontal ligament were formed in the experimental group compared to the control group. Our results support the findings of previous studies which showed that collagen materials as scaffolds can facilitate periodontal tissue regeneration^{7, 8}. Hidaka *et al.*²⁰ reported that the alkaline phosphatase activity of periodontal ligament cells was promoted in three-dimensional cell culture using collagen gel. Miyaji *et al.*¹⁴ showed that

Table 1 Histomorphometric analysis (mean $\pm$ SD)

	Control group (n = 8)	Experimental group (n = 9)
Defect height (mm)	4.76 $\pm$ 0.19	4.83 $\pm$ 0.41
New bone height (mm)	0.81 $\pm$ 0.32	1.89 $\pm$ 0.52*
New bone area (mm ²)	1.64 $\pm$ 0.70	4.45 $\pm$ 1.05*
New Cementum (mm)	0.37 $\pm$ 0.26	1.81 $\pm$ 0.75*
New periodontal ligament (mm)	0.34 $\pm$ 0.25	1.36 $\pm$ 0.58*
Ankylosis (mm)	0.0 $\pm$ 0.0	0.02 $\pm$ 0.07
Gingival connective tissue (mm)	2.97 $\pm$ 0.97	1.64 $\pm$ 0.98*
Junctional epithelium (mm)	0.90 $\pm$ 0.95	0.93 $\pm$ 0.95
Gingival recession (mm)	0.34 $\pm$ 0.52	0.11 $\pm$ 0.33

*Statistical difference (Student's t-test) compared to control group (p < 0.05).

collagen hydrogel implantation stimulated cell proliferation associated with regeneration of cementum and periodontal ligament along the root surface. Additionally, ankylosis should be inhibited by regeneration of both alveolar bone and periodontal ligament simultaneously. The present experiment using collagen hydrogel-sponge composite found little evidence of ankylosis on any part of the root surface with bone regeneration in periodontal defects.

In periodontal therapy, periodontal ligament cell and osteogenic cell repopulation are theoretically considered to accelerate functional periodontal tissue reconstruction during wound healing¹. However, this is prevented by the rapid epithelial cell downgrowth and gingival tissue growth along the root surface from the coronal portion during the early healing. It has been reported that maintenance of the periodontal regenerative space plays a major role in the success of periodontal regeneration^{1, 21}. Shimoji *et al.*¹⁰ demonstrated that implantation of FC-HAC sponge in areas of bone perforation on rat femur could maintain the bone inductive space for long periods, thus promoting bone augmentation. The collagen hydrogel-sponge composite in the present experiment contained FC-HAC sponge.

A regenerative scaffold requires rapid replacement with low cytotoxicity in the body following cell migration and tissue reconstruction in the healing site. In our experiment, most of the collagen material had disappeared by four weeks after implantation, and inflammatory cells and root resorption were rarely observed. Thus, as also shown by previous studies, collagen hydrogel-sponge composite is a biocompatible material^{10, 14, 15}. The high replacing ability of the collagen hydrogel-sponge composite may allow early periodontal tissue formation due to early exchange of space between degrading collagen material

and periodontal cells.

It was reported that collagen hydrogels possess water absorption properties; collagen hydrogels could retain water in a 20- to 200-fold range of the empty weight¹¹. A water-absorbable scaffold may be beneficial in the healing of periodontal tissue, because the regenerative space can retain tissue interstitial fluid which contains several growth factors that promote tissue healing. It is reasonable to expect that combined use of the collagen hydrogel-sponge composite and growth factors may provide a more effective tissue engineering approach for periodontal regenerative therapy. In conclusion, implantation of collagen hydrogel-sponge composite stimulated periodontal wound healing in one-wall infrabony defects in beagle dogs.

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