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ORIGINAL ARTICLE

The Effects of Collagen Hydrogel Implantation in Buccal Dehiscence Defects in Beagles

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SYNOPSIS
Type I collagen hydrogel treated by an ascorbate-copper ion crosslinking system is highly biocompatible and highly degradable in the body. The purpose of this study was to examine the effect of collagen hydrogel on periodontal wound healing in beagles. Sixty-four periodontal dehiscence type defects were created on the buccal roots of four beagles. Subsequently, collagen hydrogel was implanted in each defect in the experimental group and sutured, and, in the control group, the defect was sutured without any application of collagen hydrogel. The percentage of the length of new bone and new cementum in the experimental group was significantly greater than that in the control group at weeks 4 ($p<0.01$) and 8 ($p<0.05$). Significantly more junctional epithelium was observed in the control group than in the experimental group at weeks 2 ($p<0.01$) and 4 ($p<0.05$). These findings thus indicate that periodontal regeneration was stimulated by collagen hydrogel implantation in beagles.

***Key words**: collagen hydrogel, periodontal regeneration, scafforld, barrier*

INTRODUCTION

In periodontal regeneration, it is generally considered that functional periodontal tissue can be reestablished by accelerating periodontal ligament cell repopulation using several biological factors and materials and also by placing a local barrier that can inhibit the epithelial downgrowth. The regenerative scaffold, a major element of tissue engineering, may provide the environment and space for repopulation and specialization of the cells and extracellular matrix without any cytotoxicity. It may also act as a barrier against gingival epithelial cells, gingival connective tissue, and oral environment leaks [1].

Type I collagen, which is a natural

biomaterial that plays a major role in tissue formation, is widely used as a tissue graft [2, 3] and as a regenerative scaffold [4-8]. It has been reported that cell migration, growth, and differentiation are regulated by collagen matrix [9,10]. It was also demonstrated that collagen sponge promoted bone formation and mineralization[11]. Moreover, Type I collagen stimulates osteoblastic differentiation of bone marrow cell and osteogenesis in vitro [12,13]. The gel form has the advantage of easy application in the body, and has been employed in a number of tissue engineering studies [14, 15].

Generally, the mechanical stiffness of collagen gel is improved by chemical cross-linking with glutaraldehyde [16]. But some investigators revealed that aldehydes and other metabolites were considerably cytotoxic [17,18]. Ishikawa et al. [19] have succeeded in preparing a highly bio-safe collagen hydrogel with a different cross-linking method; in which collagen solution was incubated with ascorbate and Copper ion. With this method, the viscoelastic hydrogel acquires the property of water absorption and shows a better hemostatic effect. The collagen hydrogel also has the ability to cure rapidly in blood at neutral pH [19]. Matsui et al. [20] reported that this collagen hydrogel possessed high tissue-compatibility and high replacing ability after application, and enhanced wound healing in skin defects in rats. Therefore, application of the collagen hydrogel in periodontal defects may stimulate wound healing and regeneration not only by osteoblastic effects, but also by maintaining space with low cytotoxicity.

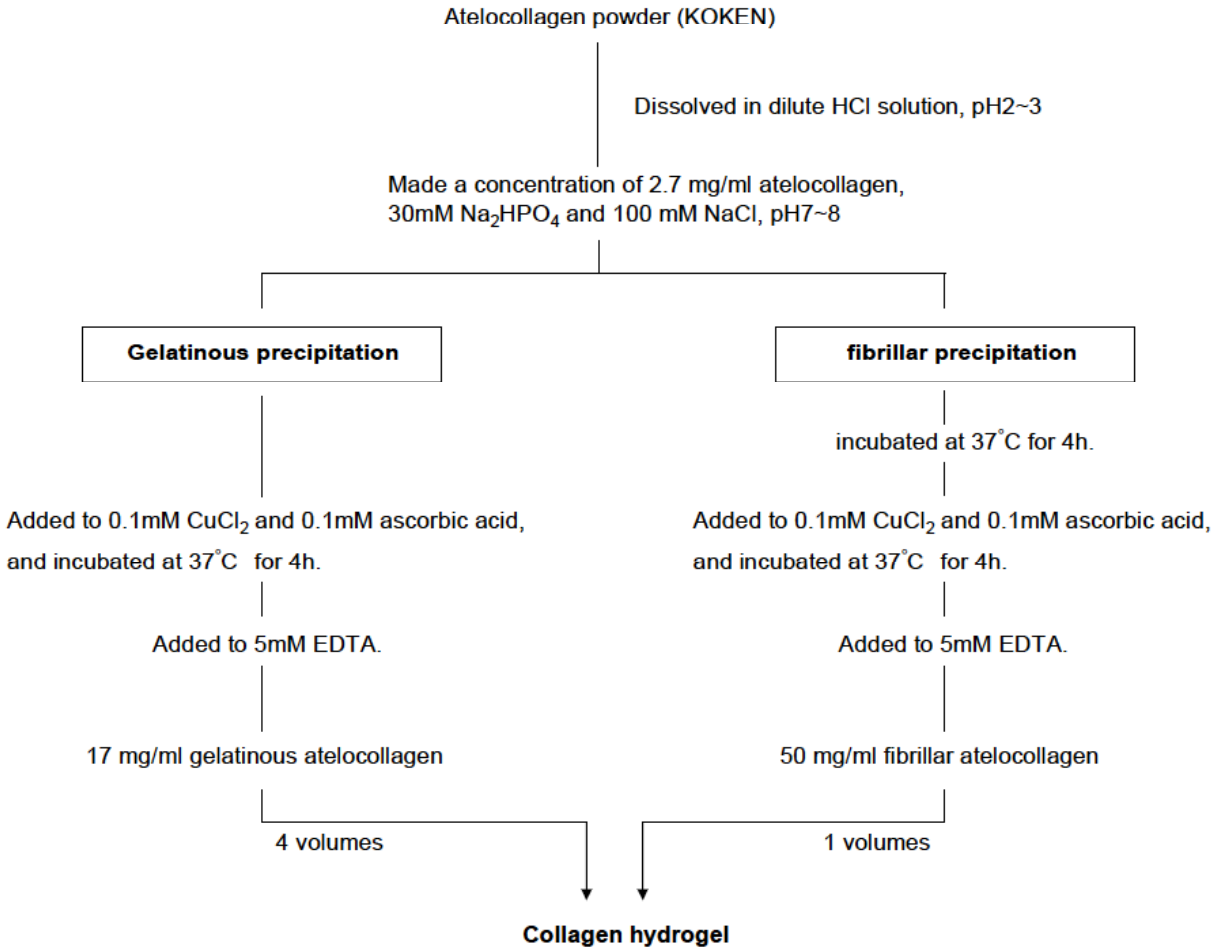

Figure 1 Preparation of collagen hydrogel.

Thus, this study was designed to evaluate the histologic and histomorphologic effects of collagen hydrogel implantation on periodontal wound healing in beagle dogs.

MATERIALS AND METHODS

Preparation of collagen hydrogel

Collagen hydrogel (Terumo, Tokyo, Japan) used in this experiment was prepared as described previously[19,20] (Fig. 1).

Animals

Four healthy female beagle dogs, 10-12 months-old, were used in this experiment. The experimental protocol (No, 01029) followed the guidelines for the care and use of laboratory animals of the Graduate School of Medicine, Hokkaido University. Surgical procedures were performed under general anesthesia with medetomidine hydrochloride (0.1 ml/kg, Domitor, Meiji seika, Tokyo, Japan) and ketamine hydrochloride (0.1 ml/kg, Ketaral 50, Sankyo, Tokyo, Japan), and local anesthesia with lidocaine hydrochloride (2% with 1:80,000 epinephrine, Xylocaine, AstraZeneca, Osaka, Japan).

Surgical procedure

Following reflection of the mucoperiosteal flap on the buccal side of the 2nd and 3rd premolars, alveolar bone to a depth of 6 mm (measured from the cemento-enamel junction) was removed using a rotating round bur under water irrigation. The root surface facing the defect was planed to remove the cementum. Reference notches indicating the cemento-enamel junction and bottom of the defect were prepared on the root surfaces. Sixty-four periodontal dehiscence type defects were thus surgically created, or 16 in each animal. Defects in alternate quadrants in dogs are then randomly assigned to the experimental and control groups.

Subsequently, the denuded root surface was demineralized with 24% EDTA (pH 7.0) for 3 min, and washed with saline. In the experimental group, collagen hydrogel was implanted in each defect, and the flap was securely sutured. In the control group, the flap was closely sutured without implantation of collagen hydrogel. The animals of both the groups received ampicillin sodium (300 mg/kg, Viccillin, Meiji seika, Tokyo, Japan) daily for 3 days and a plaque control regimen with 0.5% chlorhexidine twice weekly, for the entire period of the experiment.

Histological procedure

The animals were enthanized with an overdose of sodium pentobarbital (0.5 ml/kg, Nembutal injection, Abbott Laboratories, Abbott Park, IL, USA) following general anesthesia. Specimens were collected from the wound at weeks 1, 2, 4, and 8 post-surgery. The tissue blocks, including teeth, bone and soft tissue, were fixed in 10% formalin, decalcified in 10% formic-citric acid, and embedded along the buccolingual plane in paraffin wax. Six-μm-thick sections were serially prepared and stained with hematoxylin-eosin (HE).

Immunostaining of osteocalcin was done using a streptavidin-biotin staining kit (Histofine, Nichirei, Tokyo, Japan) and bovine monoclonal anti-osteocalcin (1:200, Takara Bio, Otsu, Japan). Dewaxed paraffin sections were incubated with 10% rabbit serum to block non-specific reactions and subsequently with an optimal solution of a primary antibody for osteocalcin up to 24 hrs at 4˚C. The sections were then incubated with biotinated secondary antibodies for 10 min, and then treated with 3,3'-diaminobenzidin. As negative control, sections were stained with phosphate buffered saline instead of the primary antibody.

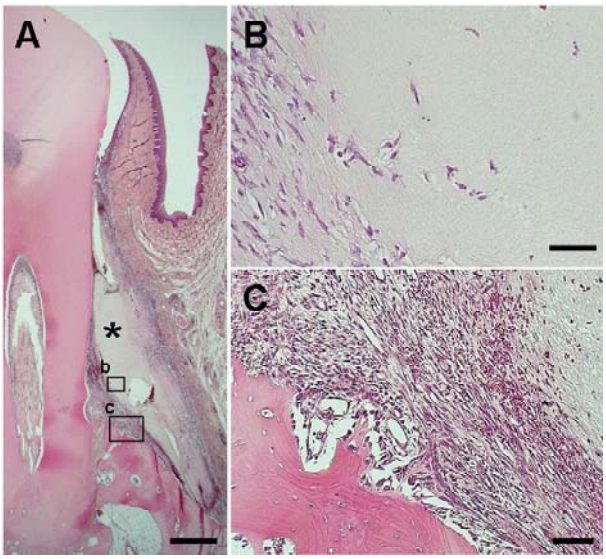

Figure 2
Experimental group at week 1. **A)** Collagen hydrogel (star) shown between the root surface and gingival flap. **B)** Higher magnification of the framed area (b) in A. Proliferation of fibroblastic cells on the inner side of the collagen hydrogel sparsely. **C)** Higher magnification of the framed area (c) in A. Osteoblastic cell proliferation was observed in the area of alveolar bone crest. (HE staining; Scale bars: A = 1mm; B = 50 μm; C = 100 μm.)

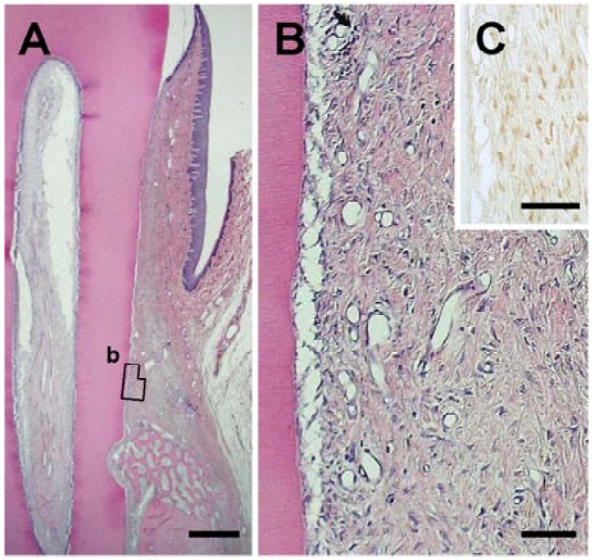

Figure 3
Experimental group at week 2. **A)** Defect area was filled with newly formed connective tissue and regenerated bone. **B)** Higher magnification of the framed area (b) in A. Numerous fibroblastic and osteoblastic cells were observed in the new connective tissue. **C)** Osteocalcin was localized on the osteoblastic cells in connective tissue. (A and B: HE staining; C: osteocalcin immunostaining; Scale bars: A = 1 mm; B and C = 100 μm.)

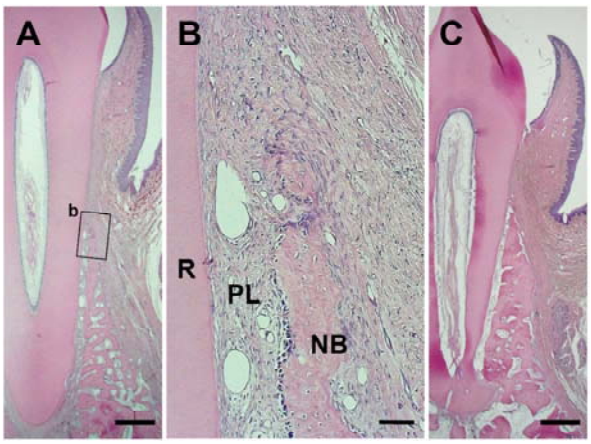

Figure 4.
Experimental groups at weeks 4 and 8. **A)** At week 4, alveolar bone regenerated in defect area extensively. **B)** Higher magnification of the framed area (b) in A. New cementum and bone formation (NB) were enhanced along the root surface (R) and periodontal ligament (PL) was reconstructed. **C)** At week 8, periodontal regeneration was observed in the defect area. (HE staining; Scale bars: A and C = 1 mm; B = 100 μm.)

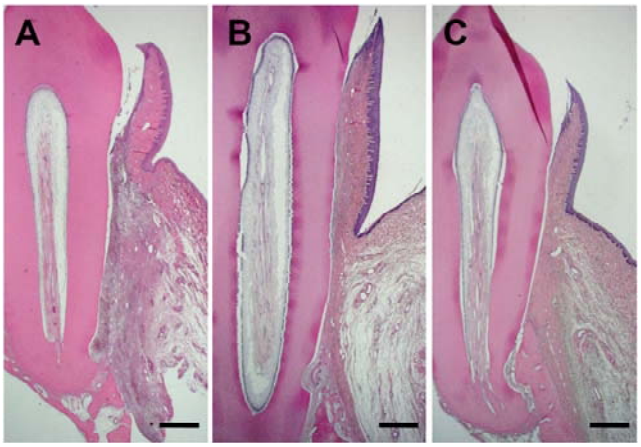

Figure. 5.
Control groups. **A)** At week 1, defect area was filled with granulation tissue. **B)** At week 2, new connective tissue was frequently thin compared to experimental group. **C)** At week 8, periodontal regeneration was demonstrated only in the apical portion of the defect. (HE staining; Scale bars: 1 mm.)

Histomorphometric analysis

Three HE-staining sections were taken; one was approximately from the center of the root, and the other two were 100 μm from either side of the center. The following four measurements were performed for each stained section under light microscope:

1. Defect height: Distance between the apical notch and the cemento-enamel junction.
2. New bone: Percentage of the length of newly formed alveolar bone in relation to the defect height.
3. New cementum: Percentage of the length of newly formed cementum on the root surface in relation to the defect height.
4. Junctional epithelium: Percentage of the length of junctional epithelial downgrowth on the root surface in relation to the defect height.

Statistical analysis

The means and standard deviations for each parameter were calculated for the experimental and control groups. Differences between the experimental group and control group were analyzed using the Mann-Whitney U test for paired observations (N = 8). p values < 0.01 and < 0.05 were considered statistically significant. All statistical procedures were performed using a software package (Stat View, Abacus Concepts, Berkeley, CA, USA).

RESULTS

Histological observations

Experimental group

At week 1, the collagen hydrogel remained between the instrumented root surface and gingival flap. Blood coagulum was scantily demonstrated in between the alveolar bone and collagen hydrogel. A newly formed connective tissue including numerous fibroblastic cells, continuous with the original periodontal ligament and alveolar bone, was was observed. Several fibroblastic cells were scattered on the inner side of the collagen hydrogel. A large number of osteoblastic cells were observed in the area of the alveolar bone crest. Few inflammatory cells were also seen in the periodontal tissue around the remnants of collagen hydrogel. (Fig. 2)

At week 2**,** the defect areas were filled with newly formed connective tissue with no remnants of collagen hydrogel. The newly formed connective tissue included osteoblastic cells expressing osteocalcin and fibroblastic cells. The cementum and alveolar bone regeneration occurred parallel to the root surface. Downgrowth of junctional epithelium was slightly observed (Fig. 3).

At week 4**,** the alveolar bone, appearing as woven bone, had extensively regenerated in the defect area and increased new cementum was observed on the dentin surface. Functionally oriented periodontal ligament tissue was also reestablished and Sharpey's fibers inserting into both the new cementum and alveolar bone were seen (Fig. 4-A, B).

At week 8, there was marked alveolar bone, cementum and periodontal ligament regeneration in the defect area. The newly formed bone was mature, and appeared similar to lamellar bone (Fig. 4-C).

Control group

At week 1, the defect area was filled with granulation tissue composed of numerous infiltrating inflammatory cells, blood vessels, and blood coagulum. Downgrowth of junctional epithelium was prominently observed along the root surface (Fig. 5-A).

At week 2, the newly formed connective tissue, which covered the treated root surface, was mostly thin and contained few fibroblastic cells compared to those of the experiment

group at the same observation period (Fig. 5-B).

At weeks 4 and 8, bone formation was observed only in the apical portion of the defect and newly formed cementum and periodontal ligament were present only in the apical notch of the root surface (Fig. 5-C).

Only mild root resorption with no ankylosis was seen in both the groups.

Histomorphometric analysis

The lengths of both the newly formed alveolar bone and cementum in the experimental group were significantly greater than those in the control group at week 4 ($p<0.01$) and week 8 ($p<0.05$). A significantly longer junctional epithelium was measured in the control group than in the experimental group at week 2 ($p<0.01$) and week 4 ($p<0.05$) (Table 1).

DISCUSSION

The present study focused on periodontal wound healing and regeneration following manual application of a new collagen hydrogel material in dehiscence type defects after demineralization of the root surface with EDTA. It was reported that 24% EDTA at neutral pH could remove the surface smear layer and expose the collagen matrix when applied to a diseased dentin surface [21]. Furthermore, an EDTA-treated dentin surface, compared to a surface etched by acid solution at low pH, appeared to be more inviting for cell proliferation and subsequent connective tissue formation [22]. Thus, in the present study, 24% EDTA was used for demineralizing the root surfaces.

Periodontal regeneration is facilitated by the early repopulation of periodontal ligament cells and osteoblasts in the wound site. However, this can be prevented by the rapid apical downgrowth of gingival epithelial cells onto the root surfaces forming the long junctional epithelium. The histological findings of this study revealed that the downgrowth of junctional epithelium was inhibited by implantation of the collagen hydrogel. The collagen hydrogel may act as a physical barrier against gingival migration, probably due to the rapid transformation of the collagen hydrogel into a stiffer form. It has been reported that collagen treatment to the root surface facilitated an anti-migratory effect on gingival epithelium in dogs [23]. Therefore, gingival epithelial cell downgrowth along the curetted root surface may be reduced by the biological sealing effect of collagen matrix in hydrogel as well as by its anti-migratory effect.

Table 1. Histomorphometric analysis of periodontal wound healing (N = 8, mean ± SD)

% New cementum	Week 1	Week 2	Week 4	Week 8
Experimental group	0.0 ± 0.0	0.0 ± 0.0	19.4 ± 18.1 †	15.4 ± 10.4 *
Control group	0.0 ± 0.0	0.2 ± 0.5	4.6 ± 3.3	4.8 ± 7.6
% New bone				
Experimental group	0.0 ± 0.0	31.1 ± 16.0	52.3 ± 10.0 †	49.3 ± 15.6 *
Control group	0.0 ± 0.0	22.4 ± 7.9	33.0 ± 12.2	32.2 ± 9.6
% Junctional epithelium				
Experimental group	9.4 ± 3.9	0.4 ± 1.1 †	7.1 ± 11.6 *	13.4 ± 20.2
Control group	12.4 ± 7.4	14.6 ± 13.9	25.3 ± 15.2	18.6 ± 18.5

* Statistical difference compared to control group ($p < 0.05$).
† Statistical difference compared to control group ($p < 0.01$).

In the histological findings for the experiment group at week 1, inflammatory cells and blood coagulum were rarely observed around the implanted collagen hydrogel, but a cell rich connective tissue continuous with the original periodontal ligament and alveolar bone was found around the collagen hydrogel. Recently, Type I collagen has been used as a scaffold in tissue engineering for various regenerative procedures [24]. The antigenicity of atelocollagen due to collagen telopeptides is suppressed by enzymatic digestion with pepsin and then used as a biomaterial in regenerative procedures. Usually, the cross-linking treatment of collagen material was performed using glutaraldehyde to increase its biostability. However, glutaraldehyde cross-linked materials are poorly biocompatible with some cell lines such as fibroblasts and osteoblasts [25, 26]. In our experiment, the regeneration of periodontal ligament and alveolar bone at the defect site induced by implantation of collagen hydrogel might suggest that the collagen hydrogel treated by alternative cross-linking with the ascorbate-copper ion system [27], has a low cytotoxic effect. The presence of minimal numbers of inflammatory cells and macrophages in the defect sites of the experimental group also confirms that the collagen hydrogel is biocompatible for regenerative procedures.

Although the implanted collagen hydrogel disappeared after two weeks, numerous fibroblasts and osteoblasts were seen in the regenerating area, suggesting that the collagen hydrogel may have the capacity of forming a scaffold for osteoblasts and periodontal ligament cells. It is considered that the regenerative scaffold requires rapid replacement with low cytotoxity in the body following cell migration and tissue reconstruction in the healing site. The high replacing ability of the collagen hydrogel may allow early periodontal tissue formation due to early exchange of space between degrading collagen hydrogel and osteoblastic and fibroblastic cells.

In the experimental group at week 2, the presence of cells in the connective tissue expressing osteocalcin, a marker of osteoblastic phenotype, supports previous findings that collagen enhanced osteoblastic differentiation [10-13]. Thus, collagen hydrogel implantation in the defect may enhance osteogenic differentiation of undifferentiated mesenchymal cells leading to alveolar bone regeneration.

Collagen hydrogel in the present study contained ascorbic acid for chemical cross-linking [19]. Another study reported that ascorbic acid may stimulate proliferation and differentiation of periodontal ligament cell in terms of DNA synthesis and ALPase activity including enhanced secretion of attachment and spreading factors [28]. Therefore, the ascorbic acid in hydrogel may play an important role in the regeneration of periodontal ligament.

It can be concluded that implantation of collagen hydrogel in the dehiscence defect may induce periodontal regeneration by acting as a physical barrier against proliferation of gingival epithelium and as a scaffold for osteoblasts and periodontal ligament cells, with high biocompatibility.

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