Vol. ■ No. ■ Month 2017

# Regeneration of gingival tissue using in situ tissue engineering with collagen scaffold

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**Objective.** The aim of the study was to evaluate 2 types of collagen scaffold for gingival regeneration.
**Study Design.** Two types of collagen scaffolds, CS-pH7.4 and CS-pH3.0, were prepared by processing atelocollagen at pH 7.4 or 3.0, respectively, followed by dehydrothermal treatment. Gingival wounds with sizes of 4 × 6 mm (rectangle) or 6 mm diameter (circle) were made with buccal incisions in beagle dogs. The defective area was surgically covered with the CS-pH7.4, CS-pH3.0, or no scaffold (control). Gingival regeneration was assessed by monitoring the differences in the lengths of the epithelial and submucosal tissues at the wound site and the normal site. Histopathologic assessments were performed by 4 evaluators independently; statistical significance was evaluated by using the Wald test.
**Results.** Significantly higher recovery of epithelial and submucosal tissues, which, in turn, resulted in recovery of gum thickness, was observed in gingival wounds treated with the CS-pH7.4 compared with that in the control. CS-pH3.0 treatment also resulted in higher gingival regeneration compared with the control; however, the effects were more pronounced in wounds treated with the CS-pH7.4. CS-pH7.4–treated wounds showed better gingival regeneration compared with the control and CS-pH3.0-treated wounds, even after adjusting for interevaluator differences using a linear mixed model.
**Conclusions.** CS-pH7.4 is a promising scaffold for gingival tissue regeneration. (Oral Surg Oral Med Oral Pathol Oral Radiol 2017;■:1–7)

Morphologic and functional reconstruction of lost or damaged gingival tissues is a major challenge in regenerative periodontal therapy.[1,2] Many gingival and periodontal diseases, tooth extraction wounds, trauma resulting from dental injury, and implantation-associated gingival volume loss or soft tissue damage require immediate attention to achieve appropriate gingival regeneration.[2-5] The final goal of periodontal therapy is to completely regenerate lost tissues; however, conventional treatment approaches are restricted to periodontal repair, which has limited effectiveness with regard to regeneration.[6] Notably, any periodontal repair that does not result in restoration of the original morphology and function of the tissue may lead to scarring, thereby having an adverse impact on aesthetics and patient satisfaction.[6-8] Therefore, it is necessary to explore effective therapeutic methods for gingival regeneration, with a focus on restoring the original morphologic, aesthetic, and functional status of the tissue.[9]

In the past few decades, many attempts have been made to develop materials that can accelerate the reconstruction of periodontal tissues.[10] Matrix-based scaffolds, stem cells, and growth factors have shown great potential for regenerative therapies.[10] In dentistry, such approaches have been explored to promote wound healing, restore periodontal ligament attachment, provide a wider zone of attached gingiva, and cover the exposed root surfaces.[2] Esposito et al. conducted a systematic review of soft tissue management for dental implants[11]; they concluded that evidence is not sufficient to provide recommendations for the optimal soft tissue augmentation technique. Several studies have reported the clinical applicability of collagen scaffolds for oral soft tissue augmentation to support wound healing.[12-14] Many of such studies have recommended the modification of collagen to improve its regenerative potential.[6,8,15-18] We recently reported the development of a weakly denatured collagen-based scaffold (CS-pH7.4), which was synthesized by processing atelocollagen at pH 7.4, followed by dehydrothermal treatment and orientation of collagen fibers; we established that this material has biocompatibility and space maintenance ability when embedded under the back skin of rats.[19,20] We hypothesize that CS-pH7.4 may also have the potential for reconstruction of gingival tissues; however, to our best

## Statement of Clinical Relevance

Tissue regeneration is required for several types of oral surgery and medical conditions. Here, we show the efficacy of weakly denatured collagen for healing gingival wounds. These findings are of high clinical significance in rapid and functional tissue regeneration.

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Received for publication Jan 25, 2017; returned for revision Apr 25, 2017; accepted for publication May 6, 2017.

2212-4403/$ - see front matter
http://dx.doi.org/10.1016/j.oooo.2017.05.471

knowledge, no reports have described the efficacy of CS-pH7.4 for gingival tissue regeneration.

In this study, we explored the suitability of CS-pH7.4 for tissue regeneration in gingival incision wounds. We also compared wound healing by CS-pH7.4 with the healing obtained by collagen scaffolds synthesized at pH 3 (CS-pH3.0). Considering the significance of possible variations in individual assessments during pathologic evaluations, all histopathologic assessments were conducted by 4 independent evaluators, and a linear mixed model was employed to compare the study groups.

## MATERIALS AND METHODS

### Materials

The scaffolds were fabricated. In brief, atelocollagen (NMP collagen PS, provided by Nippon Meatpackers, Tsukuba, Ibaraki, Japan) was used to make the collagen scaffold. This product is sold in powder form and prepared according to the manufacturer's instructions to yield the collagen mass (pH 7).

Detailed protocols for the synthesis of CS-pH7.4 have been described in our previous studies.[19,20] Briefly, a 3% w/v collagen fiber suspension in water was prepared by grating the collagen mass (pH 7) to a size of $2 \times 2 \times 2$ mm$^3$ and mixing with sterilized Milli-Q water in a Hybrid Mixer (HM-500; Keyence, Osaka, Japan) for 2 minutes, followed by cooling at 4°C for approximately 30 minutes. The procedure was repeated 5 times, and the mixture was left at 4°C for 12 hours to obtain a uniform suspension. The pH was then adjusted to 7.4 with 1 N NaOH, and the suspension was stirred at 5000 rpm for 15 minutes on ice in an Ace Homogenizer (HM-500; Nissei, Tokyo, Japan). The collagen suspension was then transferred to containers and preserved by refrigerating at −10°C for 12 hours. To obtain the appropriate orientation of collagen fibers, the cooling was performed only from the bottom of the containers.[18] The suspension was freeze-dried for 3 days.

The CS-pH3.0 scaffold was prepared using a similar procedure after adjusting the pH of the 3% w/v collagen fiber suspension to 3.

Heat-dehydration cross-linking was induced under low pressure ($1 \times 10^{-1}$ Pa) and at 140°C for 6 or 12 hours for CS-pH7.4 and CS-pH3.0 collagen, respectively. Collagen was then cut into 5-mm-thick sections, and the surface of the collagen pieces was made even by applying pressure using a smooth surface hammer.

### Animals

Adult beagle dogs (n = 9; either sex; weight: 8.0-14 kg) were used in this study. As feedstuff, dog food soaked in water was provided throughout the experimental period. The animals were healthy and showed no periodontal disease or gingival recession. All experiments were performed according to the principles of laboratory animal care advocated by the Animal Research Committee of Kyoto University (2007).

### Gingival wounds

A circular incision 6 mm in diameter was created on the buccal gingiva near the anterior teeth or molars using a gum punch or scalpel (Figure 1). Damage to the movable mucous membrane was avoided. When an incision 6 mm in diameter was not possible, the incision was created to a size of 4 × 6 mm. The CS-pH7.4 or the CS-pH3.0 scaffold was used to cover the defective region. A control group (wound without any collagen scaffold) was also used for comparison. The defective region (with or without collagen scaffold) and the surrounding area was completely covered with COE PAK (GC America Inc., Alsip, IL), which was bound to the adjacent tooth with an adhesive resin (SuperBond, Sun Medical Co, Shiga, Japan; Figure 2A).

### Histopathologic examination

At 2 weeks after treatment, the animals were euthanized with an overdose of sodium pentobarbital, and autopsy was performed. The period of 2 weeks was chosen on the basis of our preliminary experiments in control, CS-pH7.4 scaffold−treated, and CS-pH3.0 scaffold−treated gingival wounds, which suggested that a 2-week period was optimal for monitoring differences in the regenerative efficacy of collagen. Extirpation, including the sample application site, was performed using a cast cutter, and the samples were fixed in 10% neutral-buffered formalin. The material was fixed with 10% formalin (for >1 week), and decalcification was carried out at 21°C to 22°C for 3 to 4 weeks using 5% formalin formate. The samples were then sectioned to ensure that the existing alveolar bone formed a "U" shape. Paraffin-embedded sections were prepared using standard methods and then stained with hematoxylin and eosin (H&E). The recovery was evaluated by comparing the thickness of the mucosal epithelium of the gum region and the thickness of the submucosa at the healing site and surrounding tissue. Sections of the images selected by optical microscopy (BZ-9000; Keyence Corporation, Osaka, Japan) were captured and digitized on the microcomputer. Healing extents were compared on the basis of the differences in the lengths of the healed and normal parts of the gingiva. The sum of the thickness of the epithelium and the length of the submucosal tissue at the gingival

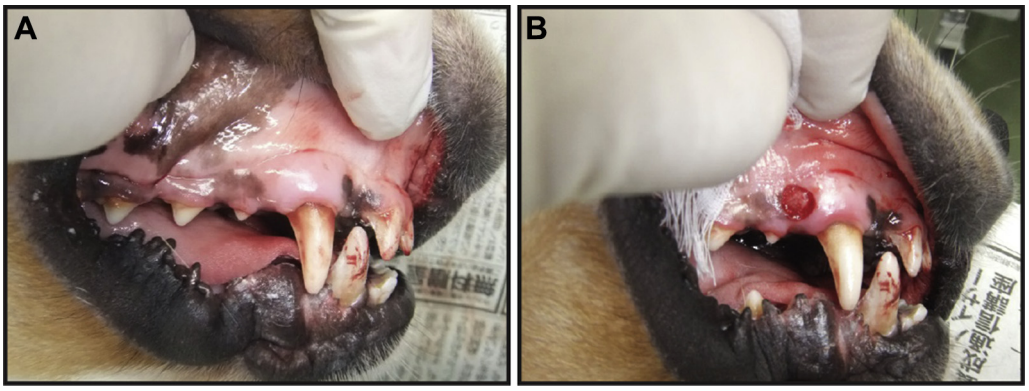


Fig. 1. Representative images showing the location of the surgical site. A circular incision 6 mm in diameter was created on the buccal gingiva at the anterior tooth region or molar region using a gum punch. The left image (A) shows the site before wounding, and the right image (B) shows the site after wounding.

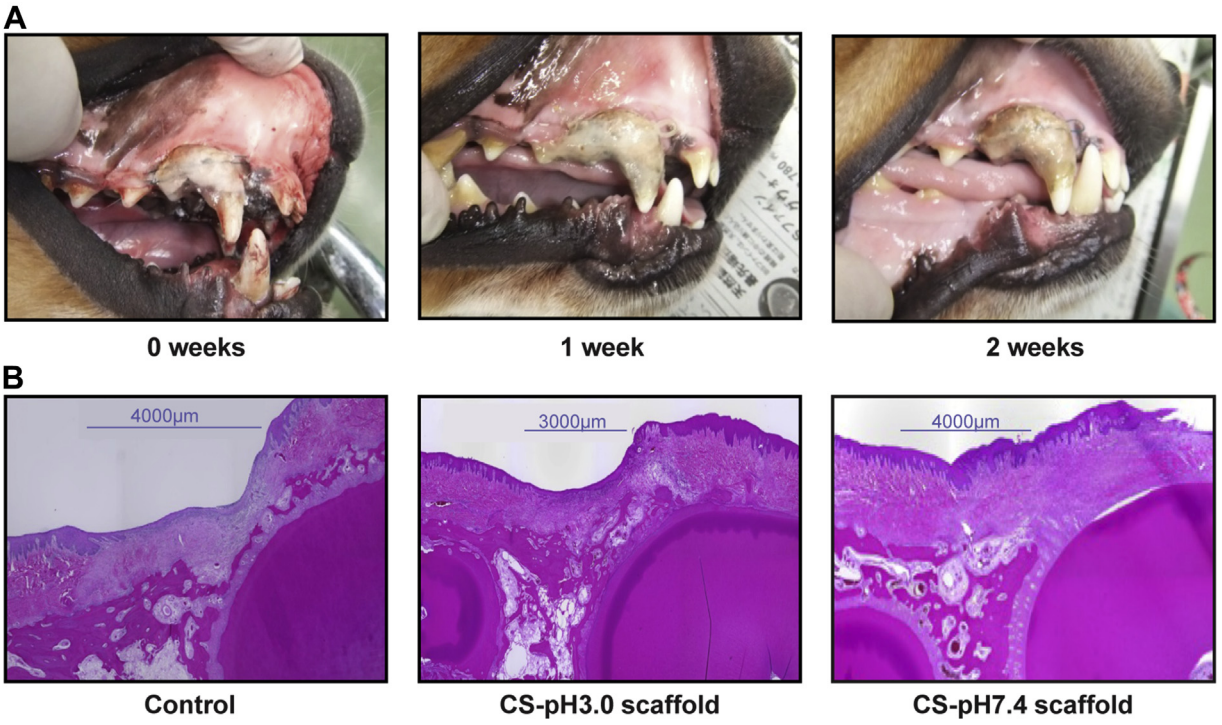


Fig. 2. (A) Wound with collagen cover at 0, 1, and 2 weeks. (B) H&E staining of the sections from control, CS-pH3.0-treated, and CS-pH7.4-treated wounds.

wound site was subtracted from the corresponding length in the normal parts of the gingival tissue to determine the extent of recovery (Supplementary Figure 1).

## STATISTICAL ANALYSIS

All data were reported as mean ± standard error (SE). A linear mixed model was constructed to evaluate the differences in the lengths of the healed and normal parts of the gingiva by the treatment and the evaluator, as described previously.[21,22] In this analysis, the model separated areas dependent only on the treatment (fixed effect) from those dependent on the evaluator (random effect); the regression formula was as follows:

$$\text{Difference in length} = (\text{Intercept}_{\text{fixed effect}} + \text{Intercept}_{\text{random effect}}) + (\text{Slope}_{\text{fixed effect}} + \text{Slope}_{\text{random effect}}).$$

Regression coefficients and their 95% confidence intervals (CIs) were estimated for all groups, and their significance was evaluated by using the Wald test. The null hypothesis was considered when there was no difference between the 2 treatment modalities, and rejection of this hypothesis was accepted when the $P$ value was $<.05$. Statistical analysis was carried out by using R statistical software (R Core Development Team, 2016).

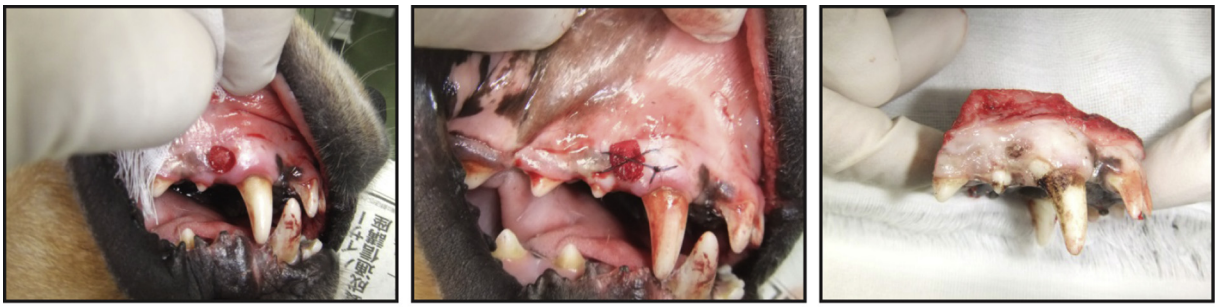


Fig. 3. Typical gross appearance of the wound (left), site after collagen treatment (middle), and healed site for a CS-pH7.4-treated gingival wounds (right).

**Table I.** Average thicknesses of the epithelial and submucosal tissues in the healing and healthy parts of the gum at weeks after collagen application

| | | *Thicknesses of epithelial and submucosal tissues at the wound site, μm* | *Thicknesses of epithelial and submucosal tissues at the normal site, μm* |
|---|---|---|---|
| *Observer* | *Treatment* | *Mean ± SD* | *Mean ± SD* |
| A | Control | 2663.0 ± 1130.8 | 4502.8 ± 1137.1 |
| | CS-pH3.0 | 3132.0 ± 624.9 | 4059.8 ± 995.0 |
| | CS-pH7.4 | 2964.7 ± 1301.3 | 4041.3 ± 823.3 |
| B | Control | 3391.4 ± 1583.0 | 4870.0 ± 997.1 |
| | CS-pH3.0 | 3395.0 ± 1445.3 | 4339.2 ± 1200.9 |
| | CS-pH7.4 | 3445.7 ± 1162.3 | 3818.4 ± 931.1 |
| C | Control | 2351.4 ± 828.1 | 4595.2 ± 1516.3 |
| | CS-pH3.0 | 3278.2 ± 980.1 | 3675.6 ± 974.5 |
| | CS-pH7.4 | 3126.1 ± 1411.2 | 3523.4 ± 756.9 |
| D | Control | 2664.4 ± 1072.8 | 3953.0 ± 1097.2 |
| | CS-pH3.0 | 3221.0 ± 1028.5 | 4076.0 ± 1130.7 |
| | CS-pH7.4 | 4053.0 ± 1764.1 | 4471.3 ± 467.4 |

## RESULTS

Figure 2A shows the integrity of a typical gingival wound covered with the collagen scaffold during the clinical course; the cover remained intact during the 2-week period, and there was no sign of damage during first or second week. Figure 2B shows H&E-stained sections of wound sites treated with the control, CS-pH3.0, and CS-pH7.4. The extent of recovery, as assessed by measuring the total thickness of the mucosal epithelium of the gum region and of the submucosa, clearly showed the positive effects of collagen administration. Tissue regeneration was not observed in the control, whereas CS-pH7.4 and CS-pH3.0 scaffold-treated wounds showed higher tissue regeneration. Figure 3 shows representative images of healing in CS-pH7.4-treated gingival wounds.

To analyze the differences between different groups and to avoid interevaluator variability in the measurement of epithelial and submucosal tissue thicknesses, we employed 4 evaluators who independently conducted the histologic analysis. Table I shows the total thicknesses of the epithelial and submucosal tissues for the wounded and normal sites of the gums at 2 weeks after surgery. Most of the evaluators found that CS-pH7.4 scaffold− and CS-pH3.0 scaffold−treated wounds showed higher total thicknesses for the epithelial and submucosal tissues compared with the control wounds. The differences between the total lengths of the epithelium and the submucosa in the healed and normal parts of the gingiva, as estimated by different evaluators for the control, CS-pH3.0, and CS-pH7.4 groups, are presented in the box chart in Figure 4.

To address evaluator-based variations in histopathologic assessments of the length between the healed portion and the normal portion of the gingiva, a linear mixed model was constructed. Additionally, to elucidate the differences in the treatment and control groups, 95% CIs of regression coefficients were estimated. The results of the linear mixed model, wherein the evaluators were introduced as a random effect, also indicated that there was a significant difference between the collagen-treated groups and the control group. The control group showed a positive intercept of 1712.7

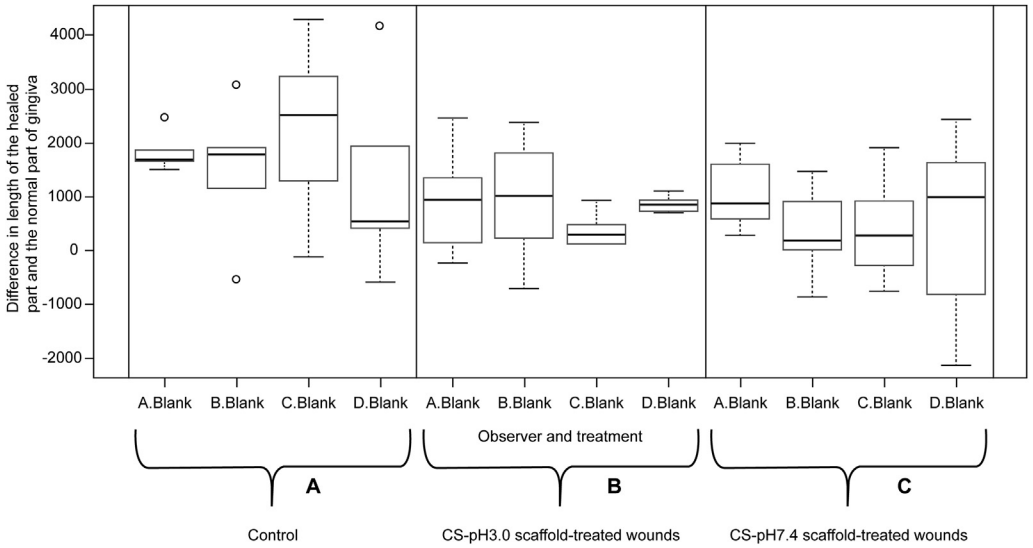


Fig. 4. Box chart showing differences in the lengths (μm) of the healed and healthy parts of the gingiva by treatment for each rater. **A**, Control. **B**, CS-pH3.0 scaffold-treated wounds. **C**, CS-pH7.4 scaffold-treated wounds.

(95% CI 1219.7-2205.7; $P < .001$), whereas the CS-pH3.0 and CS-pH7.4 groups showed negative intercepts of −931.6 (95% CI −1628.9 to −234.3; $P = .011$) and −1146.5 (95% CI −1792.0 to −500.9; $P = .001$), respectively. Although treatment with the CS-pH3.0 scaffold showed better results in terms of gingival regeneration compared with the control, CS-pH7.4 had the highest gingival regeneration, even when interevaluator differences were considered (CS-pH3.0 scaffold vs CS-pH7.4: −931.6 [95% CI −1628.9 to −234.3] vs −1146.5 [95% CI −1792.0 to 500.9]; $P = .03$).

## DISCUSSION

Our results suggested that the CS-pH7.4 scaffold facilitated tissue regeneration in gingival incision wounds. Furthermore, we established that CS-pH7.4 had a statistically significant advantage over CS-pH3.0. The results of the linear mixed model, wherein the evaluators were introduced as a random effect for the observed difference in the total length of the epithelium and the submucosa in the healed and normal parts of the gingiva, also indicated differences between the control and CS-pH7.4.

Collagen is used in several biomedical applications as a composite, blend, or chemical derivative; however, its mechanical properties and biodegradability profile needs to be tailored to suit the application.[15,16,23,24] In our previous studies, we reported the synthesis of CS-pH7.4 from collagen fiber suspensions through a process involving freeze drying and denaturation.[19,20] We also showed that the prepared CS-pH7.4 had sufficient mechanical strength to maintain the space for tissue regeneration in vivo, mainly as a result of collagen fibril orientation and mild denaturation.[19] This work confirmed the applicability of the CS-pH7.4 for gingival tissue regeneration.

pH can have a significant effect on the structural, biologic, and physicomechanical characteristics of biopolymers.[25] The pH-induced changes in the structural and supramolecular characteristics of collagen have been reported to affect the basic characteristics of collagen, including mechanical strength, porosity, and cell adhesion, thereby affecting the regenerative potential of the collagen.[13,19,26] Li et al.[27] studied the effects of pH on collagen fibrillogenesis in vitro and reported that at a low pH of 6.6, collagen molecules form small fibrils with a diameter of 85 nm; in contrast, in the pH range from 6.9 to 8.0, collagen molecules form fibrils with a diameter of approximately 200 nm.[27] Harris et al. also produced different collagen type I fibrils with in vitro fibrillogenesis of acetic acid-soluble collagen within the pH range of 2.5 to 9.0.[17] Their findings demonstrated that low pH (2.5) led to the formation of initial molecular aggregates that progressively linked together at a slightly higher pH to form subfibrils. The effects of pH and the ionic strength on the structure and stability of collagen fibrils have also been confirmed by means of X-ray and neutron diffraction techniques.[28,29] Notably, at pH 3, collagen is soluble in water, but at pH 7.4, collagen is insoluble in water and forms a turbid suspension; therefore, collagen fibers in CS-pH7.4 were thicker than those in CS-pH3.0.

In addition to pH-dependent changes in collagen, the fact that the cooling process used in the synthesis of CS-pH7.4 provided orientation of collagen fibers can

also affect the tissue regeneration efficacy of collagen scaffolds by promoting the cell transport within the scaffold.[19] We previously reported that CS-pH7.4 has optimal denaturation, cross-linking, and pore size, yielding scaffolds with superior space maintenance ability and biocompatibility.[19,20] Using scanning electron microscopy, we confirmed that this procedure yielded collagen fiber scaffolds with a specific orientation. The pore size of the scaffolds was in the range of 100 to 300 μm, and the infiltration of cells perfusing the scaffold was also significantly higher.[19] This increased infiltration of cells perfusing the scaffold is expected to facilitate tissue regeneration.[19] Furthermore, the collagen used in this work was atelocollagen, which is devoid of terminal antigenic telopeptides and thus has good biocompatibility.[19,20]

CS-pH7.4 had mechanical integrity to allow its use as a scaffold for tissue regeneration and could be easily applied on gingival wounds. Indeed, considerable efforts have been made for tailoring of the physicomechanical properties, biocompatibility, and biodegradability of collagen. Burns et al.[30] developed a novel collagen bilayer membrane and demonstrated its application in gingival recession.[31] Novel collagen matrices with fibrillated structures based on the fibrillogenesis/gelling method have been developed recently, allowing for the harnessing of the regenerative potential of collagen.[32] For periodontal tissue reconstruction, Goissis et al. treated collagen with glutaraldehyde to yield different cross-linking densities and claimed that the cross-linked collagen had the potential for periodontal tissue regeneration.[33] Compared with such efforts on the synthesis of collagen scaffolds, our synthesis approach has the major advantage of being simple and not involving the addition of any cross-linkers or fillers. Our approach, indeed, required only proper pH, orientation of collagen fibers, and dehydrothermal treatment.

Overall, our findings indicated that collagen scaffolds could promote tissue regeneration in wounds and that the pH used during the processing steps could affect the regenerative properties of collagen. However, the mechanism for accelerated localized wound healing is expected to be multifactorial, including collagen-facilitated invasion of histiocytes, fibroblasts, and blood capillaries, which promote healing.[1,9,10,15,16,34] Further studies are needed to investigate these mechanisms.

## CONCLUSIONS

This study revealed that the application of CS-pH7.4 was effective for healing gingival wound defects in beagle dogs. At 2 weeks after treatment, histologic analysis indicated higher total thickness of the epithelium and the submucosa in gingival wounds treated with CS-pH7.4 that in control wounds, reflecting the high tissue-regeneration capacity of collagen scaffolds. A linear mixed model with 4 evaluators as a random effect confirmed the higher tissue regeneration in gingival wounds treated with CS-pH7.4 than that in control or CS-pH3.0 scaffold-treated wounds. Additional studies are needed to validate these findings and extrapolate the results to humans.

Editorial support, in the form of medical writing based on authors' detailed directions, collating author comments, copyediting, fact checking, and referencing, was provided by Cactus Communications.

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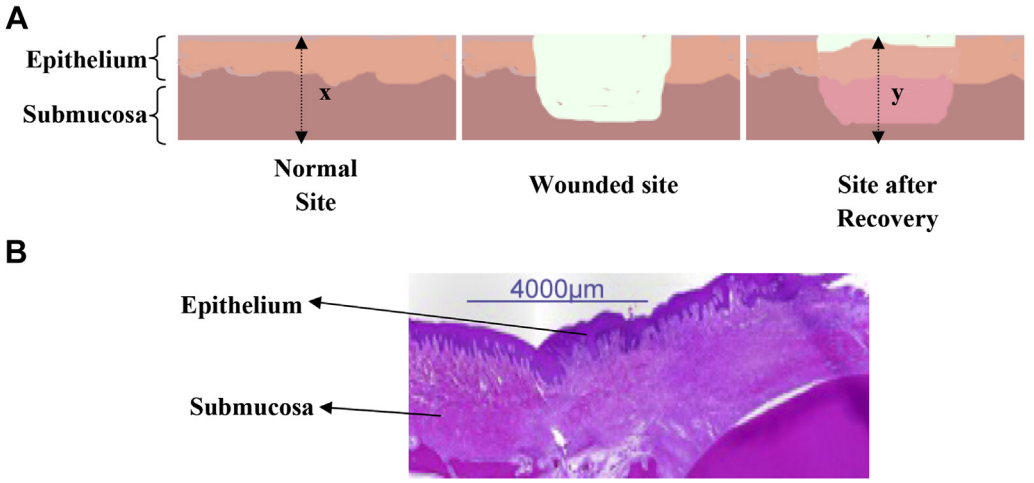
## SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.oooo.2017.05.471.




Supplementary figure 1. (**A**) Schematic, not to scale drawing, showing a normal gingival site, wounded site and site after recovery and thickness measurement (**B**) Real specimen and identification of epithelium and submucosa.