Periodontal wound healing/regeneration of two-wall intrabony defects following reconstructive surgery with cross-linked hyaluronic acid-gel with or without a collagen matrix: a preclinical study in dogs

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Objectives: In-vitro data have shown that cross-linked hyaluronic acid (HA) enhances the proliferative and migratory properties of cells involved in periodontal wound healing/regeneration, stabilizes the blood clot, reduces the inflammatory response, and facilitates angiogenesis. The aim of this study was to histologically evaluate the effects of cross-linked HA alone or combined with a collagen matrix (CM) on the periodontal wound healing/regeneration in intrabony defects. **Method and materials:** Two-wall intrabony defects (5 mm wide, 5 mm deep) were surgically created at the distal and mesial aspects of mandibular premolars in six beagle dogs. The 24 defects were randomly treated as follows: open flap debridement (OFD) + HA, OFD + CM, OFD + HA + CM (HA/CM), and OFD alone (control). At 2 months, the animals were euthanized for histologic evaluation. **Results:** The HA (2.43 ± 1.25 mm) and HA/CM (2.60 ± 0.99 mm) groups yielded statistically significantly ($P < .05$) greater formation of new attachment (ie, linear length of new cementum adjacent to newly formed bone, with inserting collagen fibers) compared with the OFD (0.55 ± 0.99 mm) group. Among the four treatment groups, the HA/CM group demonstrated the highest amount of regenerated tissues, although no statistically significant differences in any of the histometric parameters were observed between the HA and HA/CM groups. **Conclusion:** Within their limits, it can be concluded that cross-linked HA alone or combined with CM promotes periodontal wound healing/regeneration in two-wall intrabony defects in dogs. *(Quintessence Int 2021;52:2–10; doi: 10.3290/j.qi.b937003)*

Key words: animal study, biomaterials, hyaluronic acid, intrabony defect, periodontal wound healing/regeneration

Periodontitis is an inflammatory disease characterized by destruction of the tooth-supporting periodontal tissues (ie, gingiva, alveolar bone, periodontal ligament, and root cementum).[1] In addition, severe forms of periodontitis are considered to be the sixth most prevalent disease of mankind,[2] and, if left untreated, can lead to tooth loss. In the vast majority of cases, nonsurgical periodontal therapy consisting of supra- and subgingival scaling accompanied by meticulous plaque control can lead to successful resolution of the infection thus arresting disease progression and preventing its recurrence. However, in some cases, residual periodontal pockets of > 5 mm still persist following completion of active periodontal therapy and may increase the risk for disease progression (ie, further loss of attachment) and tooth loss.[3,4] These residual pockets are frequently related to the presence of intrabony periodontal defects, which have been demonstrated to negatively affect long-term tooth prognosis,[5] thus require further treatment. Today, it is generally accepted that deep intrabony defects can be successfully treated by means of periodontal surgery in conjunction with various regenerative materials such as bone grafts, guided tissue regeneration, enamel matrix derivative, growth factors, or various combinations thereof.[6-9]

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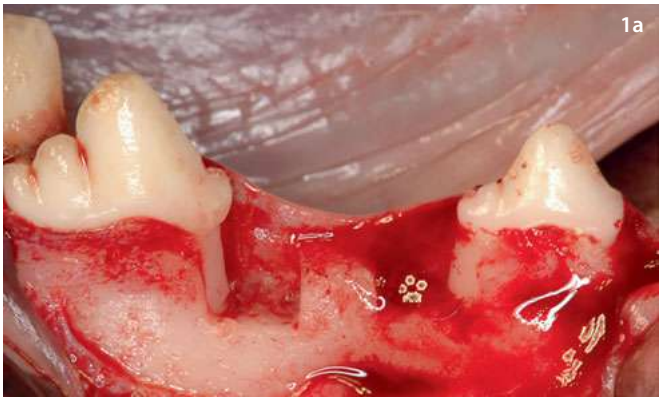
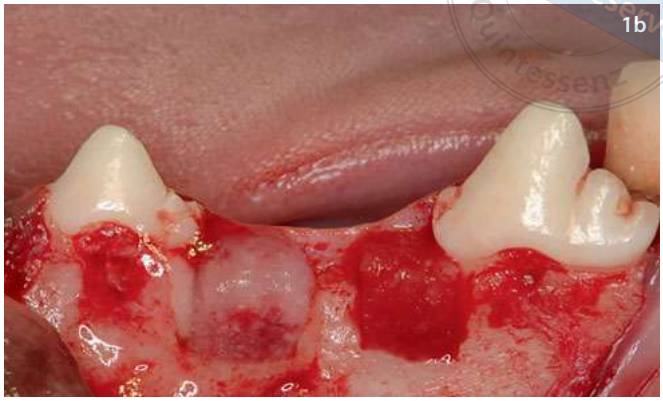




Figs 1a and 1b Clinical photographs showing the surgically created and treated two-wall intrabony defects. *(a)* Left: open flap debridement (OFD), right: hyaluronic acid (HA) gel application. *(b)* Left: HA gel and collagen matrix (CM) construct (HA/CM) was placed into the defect, right: CM placement.

Hyaluronic acid (HA) is a major natural carbohydrate component of the extracellular matrix in many tissues such as skin, joints, eyes, and periodontium,[10,11] and has unique physiochemical and biologic properties including hygroscopic,[11,12] viscoelastic,[12] bacteriostatic,[10,12,13] anti-inflammatory,[10,12,14,15] anti-edematous,[10,12] and, is in certain situations, even of osteoinductive[15,16] nature. Furthermore, extensive studies have demonstrated that HA significantly stimulates clot formation,[11,17] induces angiogenesis,[11,18] increases osteogenesis,[11,19] and plays various pivotal roles in cell adhesion, migration, and differentiation mediated by various HA binding proteins and cell-surface receptors.[20] Recent results[21,22] from a series of in vitro experiments have demonstrated that HA is fully biocompatible and does not elicit any negative effects on the viability of human periodontal ligament fibroblasts (HPFs) and human gingival fibroblasts (HGFs) but is able to increase the proliferative and migratory abilities of both cell types. Moreover, HA also triggers the expression of collagen type III alpha 1 (COL3A1) and transforming growth factor beta 3 (TGFB3), which are strongly associated with scarless wound healing; upregulates the expression of genes encoding platelet-derived growth factor B (PDGFB), basic fibroblast growth factor (FGF-2), and epidermal growth factor (EGF), essential growth factors in wound healing; and induces the expression of pro-inflammatory cytokines, which strongly influences extracellular matrix (ECM) remodeling.[21,22] Very recently, it has been demonstrated that HA strongly induces the growth of osteoprogenitors and maintains their stemness, thus potentially regulating the balance between self-renewal and differentiation.[23] Hence, it has been suggested that HA represents an ideal material to facilitate periodontal wound healing/regeneration in various types of periodontal defects. When applied in intrabony defects, HA has demonstrated successful outcomes in terms of clinical attachment level gain and probing depth reduction.[10,24-26]

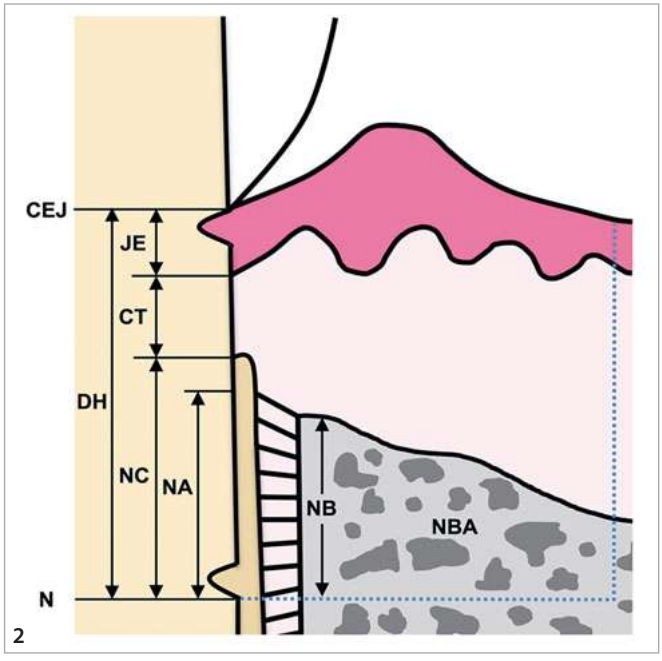
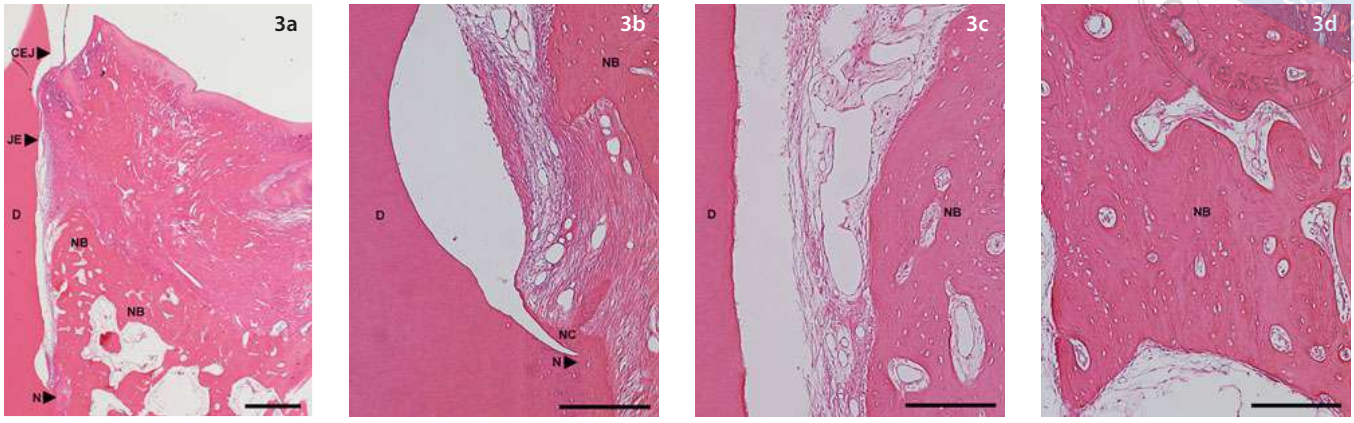


Fig 2 Landmarks/parameters used in histomorphometric analysis. The template (blue dotted line) serves as a proxy for the original defect area (5 × 5 mm). CEJ, cementoenamel junction; CT, connective tissue adhesion; DH, defect height; JE, junctional epithelial length; N, apical notch; NA, new attachment length; NB, new bone length; NBA, new bone area; NC, new cementum length.



Figs 3a to 3d *(a)* Histologic overview of defect treated with open flap debridement (control group) (scale bar, 1 mm; h&e stain). *(b)* Higher magnification of the apical portion of the defect (scale bar, 200 µm; h&e stain). *(c)* Higher magnification of the coronal portion of the bone crest (scale bar, 200 µm; h&e stain). *(d)* Higher magnification of the middle portion of the newly-formed bone (scale bar, 200 µm; h&e stain). CEJ, cementoenamel junction; D, root dentin; JE, apical end of junctional epithelium; N, apical notch; NB, new bone; NC, new cementum.

However, due to its fluid consistency, HA, similarly to other fluid biologics, may not possess sufficient space-making potential, which in turn may lead to a collapse of the mucoperiosteal flap and subsequently limit the outcomes of regenerative surgery.[6,27]

Recently, various types of collagens matrices (CM) have been successfully used as soft tissue alternatives[28,29] and/or carriers[29,30] for bioactive agents in periodontal plastic/regenerative procedures. Additionally, experimental studies in animals have shown that collagen matrices may also stabilize the blood clot and prevent flap collapse thus maintaining the space needed for the regeneration process in recession[28] and intrabony periodontal defects.[30] However, to the best of the present authors' knowledge, no study has histologically evaluated the potential effects of HA either alone or combined with CM in promoting periodontal wound healing and regeneration in intrabony defects. Thus, the present study aimed to histologically evaluate the effects of HA with or without CM on periodontal wound healing/regeneration in two-wall intrabony defects in dogs.

Method and materials

Animals

Six healthy male beagle dogs, 16 to 20 months of age and weighing 9 to 14 kg, were used in this study. The animals were housed and monitored daily for the duration of the study in the Animal Experimentation Facility Shin Nippon Biomedical Laboratories, Kagoshima, Japan. They were kept in individual cages at 20°C to 26°C, relative humidity of 30% to 70%, and a 12-hour light/dark cycle. Approximately 300 g of solid food (NVE-10, Nippon Pet Food) was provided to each animal daily and water was available ad libitum. All procedures during the in-life phase were approved by the ethical committee of the Animal Research Center of Kagoshima University, Japan (Project Approval No. D18016; Date of approval: 16 August 2018). This study conformed to the ARRIVE guidelines for preclinical animal studies.

Surgical protocol

All surgical procedures were performed under general and local anesthesia using aseptic routines by one experienced surgeon (YS). Before surgical procedures, analgesics (buprenorphine hydrochloride, 0.1 mL/kg; Leptan, Otsuka Pharmaceutical) and antibiotics (penicillin G procaine and dihydrostreptomycin sulfate aqueous suspension for injection, 0.05 mL/kg; Mycillin Sol Meiji for veterinary use, Meiji Seika Pharma) were administered intramuscularly (IM). General anesthesia was achieved with a combination of pentobarbital sodium (Somnopentyl, 0.2 mL/kg intravenously [IV]; Kyoritsu Seiyaku Corporation) and medetomidine hydrochloride (Domitor, 0.08 mL/kg IM; Orion Corporation) at a level to maintain spontaneous breathing. Local anesthesia was achieved with lidocaine HCl/epinephrine (2%, 1:80,000 Xylocaine; Fujisawa). The bilateral mandibular third premolars were carefully extracted to provide enough space

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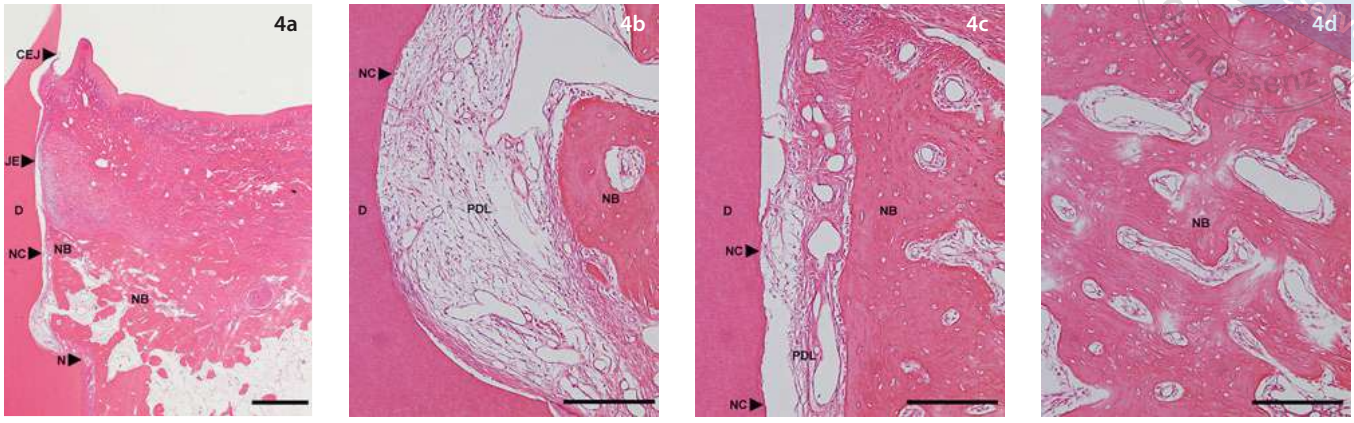


Figs 4a to 4d *(a)* Histologic overview of the defect treated with open flap debridement and collagen matrix (CM group) (scale bar, 1 mm; h&e stain). *(b)* Higher magnification of the apical portion of the defect (scale bar, 200 µm; h&e stain). *(c)* Higher magnification of the coronal portion of the bone crest (scale bar, 200 µm; h&e stain). *(d)* Higher magnification of the middle portion of the newly formed bone. CEJ, cementoenamel junction; D, root dentin; JE; junctional epithelium; N, pical notch; NB, new bone; NC, new cementum; PDL, periodontal ligament.

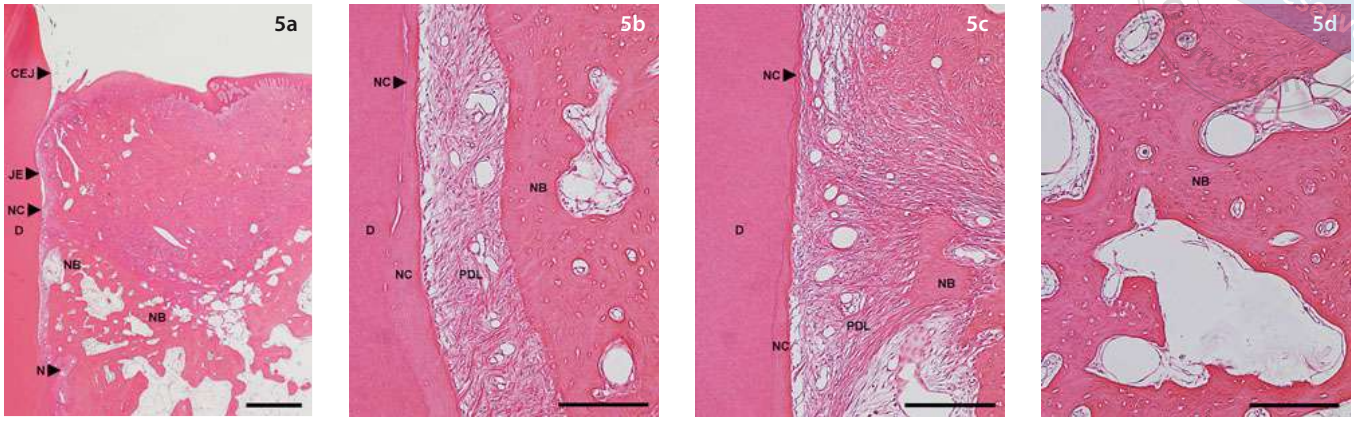
for creation of defects and for flap management to allow wound closure with primary intention healing. After a 10-week healing interval, two-wall intrabony defects (5 mm wide and 5 mm deep) were prepared bilaterally at the mesial aspect of the mandibular fourth premolars (P4) and at the distal aspect of the mandibular second premolars (P2) (four defects per dog). Following elevation of the mucoperiosteal flap, defects were created by using fissure burs with a sterile saline coolant (Fig 1). Cementum was removed using Gracey curettes and a chisel. Reference notches were made with a no. 1 round bur on the root surface at the base of the defects, at the cementoenamel junction (CEJ), and on the crown surface, to indicate the precise center plane of the intrabony defects and allow for optimal histomorphometric analysis. The 24 bilateral mandibular two-wall intrabony defects randomly received one of the following treatments after surgical debridement: cross-linked porcine collagen matrix (Fibro-Gide, Geistlich Pharma) alone (CM), cross-linked hyaluronic acid gel (hyadent BG, Regedent) alone (HA), HA + CM (HA/CM), and open flap debridement (OFD) only as a surgical control (Fig 1). In the CM group, CM was mixed with sterile saline before being applied to the defects (Fig 1b). The HA gel was applied to the root surfaces, and the defects were filled up to the adjacent alveolar crest in the HA group (Fig 1a). In the HA/CM group, the CM was fully saturated with HA and the constructs were allowed to rest for 10 minutes. The constructs were then filled in the defects with moderate pressure (Fig 1b). Maximum care was taken during surgery to prevent mixing of HA to the other site in the same side of the mandible. A periosteal releasing incision was made to allow coronal displacement of the flap, followed by suturing (Gore-Tex CV-6 Suture; W L Gore and Associates) slightly coronal to the CEJ. Sutures were removed after 2 weeks.

Postsurgical protocol

The animals were fed a soft diet for 2 weeks postoperatively. Ketoprofen for analgesia (Capisten IM 50 mg, 2 mg/kg, 0.1 mL/kg; Kissei Pharmaceutical) and an antibiotic (Mycillin Sol) were administered daily for 2 days. Plaque control was maintained with routine (3 times a week) flushing of the oral cavity with 2% chlorhexidine gluconate solution for 8 weeks after surgery.

Histologic preparation

Eight weeks after surgery, the animals were euthanized with an overdose of sodium thiopental. All defects were then resected, together with the surrounding soft and hard tissues. The tissue blocks were fixed in 10% buffered formalin, trimmed according to intraoral radiographs and the reference notch on the crown, and rinsed in phosphate-buffered saline. The samples were decalcified in Kalkitox (Wako Pure Chemical Industries), dehydrated, and embedded in paraffin. Serial 6-µm-thick sections were then prepared along the mesiodistal plane and were stained with hematoxylin and eosin (h&e).



Figs 5a to 5d *(a)* Histologic overview of the defect treated with open flap debridement and hyaluronic acid gel (HA group) (scale bar, 1 mm; h&e stain). *(b)* Higher magnification of the apical portion of the defect (scale bar, 200 µm; h&e stain). *(c)* Higher magnification of the coronal portion of the bone crest (scale bar, 200 µm; h&e stain). (d) Higher magnification of the middle portion of the newly formed bone (scale bar, 200 µm; h&e stain). CEJ, cementoenamel junction; D, root dentin; JE, junctional epithelium; N, apical notch; NB, new bone; NC, new cementum; PDL, periodontal ligament.

Histomorphometric analysis

All specimens were analyzed under a light microscope (BX51; Olympus) equipped with a computerized image system (WinROOF2015; Mitani Corporation). For histomorphometric analysis, three sections approximately 90 µm apart were selected from the most central area of each two-wall defect, identified by the length of the root canal and the reference notches. The mean value of each histomorphometric parameter was then calculated for each site. The following parameters (Fig 2) were measured by a single experienced blinded examiner (TI).

- Defect height (DH): distance between the apical extent of root planing and the CEJ.
- Junctional epithelium length (JE): distance between the apical extension of the junctional epithelium and the CEJ.
- Connective tissue adhesion (without cementum)(CT): distance between apical extent of the junctional epithelium and the coronal extent of the newly formed cementum.
- New bone length (NB): distance between the apical extent of root planing and the coronal extent of newly formed alveolar bone along the root surface.
- New bone area (NBA): newly formed trabecular bone within a template (5 × 5 mm) that served as a standardized proxy for the defect site. The template was aligned parallel to the root surface interfacing the apical extension of the root planing.[31]
- New cementum length (NC): distance between apical extent of root planing and coronal extent of newly formed cementum on the denuded root surface.
- New attachment length (NA): linear length of the root surface covered by NC adjacent to newly formed bone, with functionally oriented collagen fibers.
- Periodontal ligament score (PDL score): which was obtained by grading the periodontal ligament with the reported scoring system outlined by Wikesjö et al.[32]

Statistical analysis

The animal was chosen as the unit for the statistical analysis. The means and standard deviations (SDs) for each parameter were calculated for each of the four treatment groups. The mean scores were used to test for differences among the experimental treatments using one-way analysis of covariance and a post-hoc (Bonferroni) test for multiple comparisons. A *P* value of $< .05$ was considered statistically significant. All calculations were performed with statistical software (BellCurve for Excel; Social Survey Research Information).

Results

Clinical observations

Postoperative clinical healing was uneventful at all 24 sites. No visible adverse reactions, including suppuration, abscess for-

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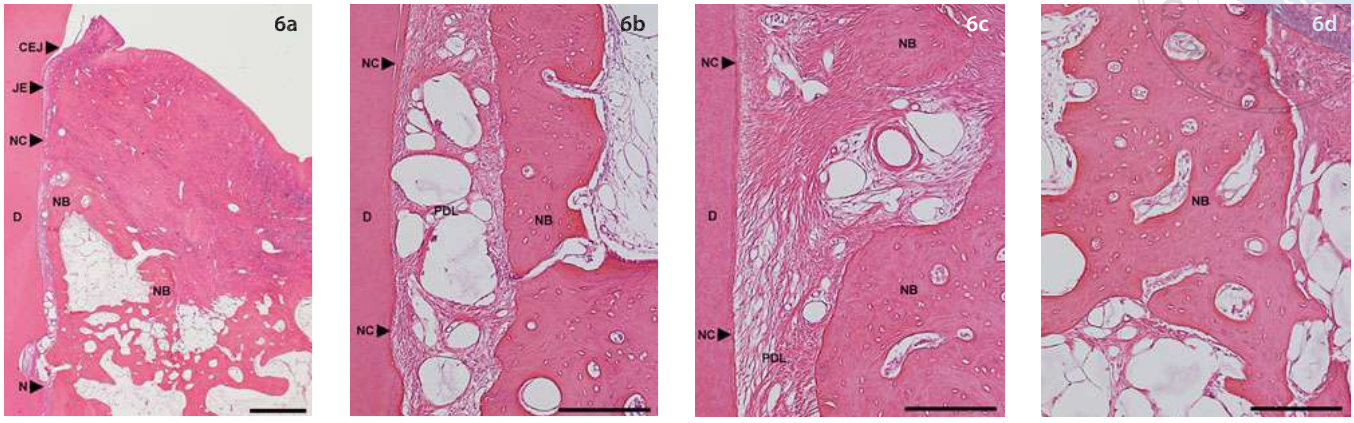

Figs 6a to 6d *(a)* Histologic overview of the defect treated with open flap debridement, hyaluronic acid gel, and collagen matrix implantation (HA/CM group) (scale bar, 1 mm; h&e stain). *(b)* Higher magnification of the apical portion of the defect (scale bar, 200 µm; h&e stain). (c) Higher magnification of the coronal portion of the bone crest (scale bar, 200 µm; h&e stain). (d) Higher magnification of the middle portion of the newly formed bone (scale bar, 200 µm; h&e stain). CEJ, cementoenamel junction; D, root dentin; JE, junctional epithelium; N, apical notch; NB, new bone; NC, new cementum; PDL, periodontal ligament.

mation, or increased tooth mobility, were observed throughout the entire experimental period.

Descriptive histology

In the OFD group, spontaneous bone formation occurred to some extent (Figs 3a and 3d). New cementum formation was restricted below the bone crest in most sites (Figs 3a and 3b). The area of connective tissue adjacent to the root surface without cementum formation was broader in the OFD group (Figs 3a and 3c) than in the other three treatment groups. In addition, new attachment formation was minimal at the level of the apical notch (Fig 3b). In the CM group, the CM was considerably resorbed and mild new bone apposition without extensive proximal host bone resorption was observed (Figs 4a and 4d). New cellular/acellular cementum was seen, with or without collagen fibers oriented parallel to or detached from the root surfaces (Figs 4a to 4c). The formation of new bone and new cementum was apparent and comparable in the HA and HA/CM treatment sites (Figs 5 and 6). New bone formation extended from the host bone toward the coronal region of the defects (Figs 5a and 6a). Newly formed bone was characterized by cancellous bone, which consists of a network of bony trabeculae containing bone marrow, blood vessels, osteoblast-like cells, and osteocyte-like cells (Figs 5a, 5d, 6a and 6d). New cellular/acellular cementum, with inserting collagen fibers running perpendicular to the root surfaces, was observed covering two-thirds of the defect area (Figs 5a to 5c, and 6a to 6c). The highly vascularized and dense new periodontal ligament-like tissue, which was formed between the new cementum and new bone, maintained its width up to the coronal portion in the HA and HA/VM groups (Figs 5a to 5c, and 6a to 6c). Neither root resorption nor ankylosis was observed in any of the defects.

Histomorphometric analysis

The results of histomorphometric analysis are shown in Table 1. No statistically significant differences were detected among the groups in regard to the following measurements (DH, JE, and CT). Nevertheless, the length of CT (without cementum formation) in the HA/CM group was the smallest and in the OFD group the greatest. For new bone length there were no significant differences among the groups examined. However, the HA/CM group showed the greatest amount of newly formed bone. The length of new cementum in the HA (3.20 ± 1.29 mm) and HA/CM (3.70 ± 1.02 mm) groups was about twice as great as in the OFD (1.77 ± 1.39 mm) group, although without statistically significant differences. The HA (2.43 ± 1.25 mm) and HA/CM (2.60 ± 0.99 mm) groups yielded statistically significantly ($P < .05$) greater formation of new attachment (ie, linear length of NC adjacent to newly formed bone, with functionally oriented collagen fibers) compared with the OFD (0.55 ± 0.99 mm) group. Moreover, the PDL scores in the HA (4.00 ± 0.89) and HA/CM (3.94 ± 0.82) groups were statistically significantly ($P < .01$) higher than that in the

Table 1 Histomorphometric parameters according to treatment modality (mean ± SD, n = 6 animals, n = 24 sites)

Parameter	Treatment group			
	OFD (n = 6)	CM (n = 6)	HA (n = 6)	HA/CM (n = 6)
Defect height (mm)	5.28 ± 0.27	5.48 ± 0.57	5.21 ± 1.05	5.18 ± 0.62
Junctional epithelium length (mm)	1.51 ± 0.91	1.77 ± 0.67	1.24 ± 0.69	1.14 ± 0.29
Connective tissue adhesion (without cementum) (mm)	1.69 ± 1.88	1.21 ± 1.09	0.73 ± 0.66	0.35 ± 0.25
New cementum length (mm)	1.77 ± 1.39	2.31 ± 1.06	3.20 ± 1.29	3.70 ± 1.02
New attachment length (mm)	0.55 ± 0.99	1.49 ± 0.69	2.43 ± 1.25*	2.60 ± 0.99*
New bone length (mm)	2.39 ± 0.69	2.45 ± 0.43	2.57 ± 1.20	2.63 ± 0.95
New bone area (newly formed trabecular bone within a 5 × 5-mm template); (mm^2)	3.01 ± 1.75	4.01 ± 1.89	3.91 ± 3.10	4.74 ± 1.76
Periodontal ligament score (1–5)	2.16 ± 1.00	3.05 ± 0.49	4.00 ± 0.89**	3.94 ± 0.82**

*$P < .05$, significantly different form the OFD group.
**$P < .01$, significantly different form the OFD group.
CM, collagen matrix; HA, hyaluronic acid gel; OFD, open flap debridement.

OFD group (2.16 ± 1.00). No significant differences in any of the histomorphometric parameters were observed between the HA and HA/CM groups.

Discussion

The present study histologically evaluated the effects of cross-linked HA with or without CM on periodontal wound healing/regeneration in two-wall intrabony defects in dogs. It was demonstrated that sites treated with HA had statistically significantly greater amounts of new attachment formation than sites treated without HA (OFD and CM groups). In other words, at the HA-treated sites, dense functionally oriented collagen fibers with many blood vessels were observed between the newly formed cementum and the newly formed bone showing high PDL scores (Table 1). More new cementum formation was also observed in the HA-applied groups compared to the OFD and CM groups, although there were no statistically significant differences. The favorable outcomes in terms of formation of cementum and periodontal ligament following HA application are in line with previous findings from in vitro studies,[21,22] which indicate that HA maintains the viability of HPFs and HGFs and increases the proliferative and migratory abilities of these cells. Furthermore, HA also triggers or upregulates the expression of a number of cytokines such as COL3A1, TGFB3, PDGFB, FGF-2, and EGF, which strongly modulate wound healing and induce angiogenesis and extracellular matrix (ECM) remodeling[21,22] as well as osteogenic differentiation.[21] Interestingly, the HA used in the present study strongly induces the growth of osteoprogenitors and maintains their stemness, thus potentially regulating the balance between self-renewal and differentiation capabilities as well.[23]

Bone formation occurred toward the coronal region of the intrabony defects in all of the groups without statistically significant differences in NB and NBA measurements. However, previous studies have shown that HA significantly induced earlier bone deposition, resulting in a more organized/mineralized bone compared to clot alone in extraction sockets in experimental animals,[33,34] by promoting the expression of bone morphogenetic protein-2, osteopontin,[33] and alkaline phosphatase activity.[35] This somewhat contradictory finding regarding the effect of HA and the outcomes in terms of bone formation in this study may be attributed to the different types of different species, or acute types of two-wall intrabony defects in dogs, whose turnover rate of bone remodeling is reported to be approximately four times faster than in humans.[36] An interesting observation in the present study was the highest amount of regenerated tissues (NC, NA, NB, and NBA) observed in the HA/CM. Taken together, these findings suggest that the cross-linked CM used in the present study was able to prevent flap collapse in the defect, thus ensuring additional space for blood clot formation and subsequent regeneration. Additionally, it may also be speculated that the CM may be capable of adsorbing HA and acting as a carrier for HA, thereby enhancing the local availability and

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effects of HA. However, these hypotheses need confirmation because no statistically significant differences were detected between HA and HA/CM groups in any of the histomorphometric parameters in the present study. Nevertheless, the results of this study provide histologic evidence which indicates that HA plays a major role in stimulating periodontal wound regeneration. Further investigations are needed to clarify the binding and release kinetics of HA when used in combination with CM.

Conclusion

In conclusion, the present study suggests that the use of HA with/without CM effectively promotes periodontal regeneration in two-wall intrabony defects in dogs, thus warranting further clinical investigation.

Acknowledgments

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Declaration

The authors declare that they have no conflict of interest.

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