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Cross-linked hyaluronic acid-gel with or without a collagen matrix in the treatment of class Ⅲ furcation defects: A histologic and histomorphometric study in dogs

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Running title: Periodontal regenerative effect of hyaluronic acid gel

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Author's contribution

Conceptualization, Yo.S. and A.S.; methodology, Yo.S. and A.S.; validation, Yo.S.; formal analysis, T.N.; investigation, Yo.S., T.I., T.N., Yu.S., M.I., and F.S; data curation, T.N. and T.I.; writing—original draft preparation, Yo.S. and A.S.; writing—review and editing, all authors.; visualization, Yu.S.; supervision, K.N.; project administration, Yo.S.; funding acquisition, Yo.S. All authors have read and agreed to the published version of the manuscript.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and all procedures performed in studies involving animals were in accordance with the ethical standards of the ethical committee of the Animal Research Center of Kagoshima University, Japan (Approval No. D20010).

Informed consent

For this type of study, formal consent is not required.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Aim

To histologically evaluate the effects of cross-linked hyaluronic acid (xHyA) with or without a collagen matrix (CM) on periodontal wound healing/regeneration in class Ⅲ furcation defects in dogs.

Materials and methods

Class Ⅲ furcation defects were surgically created in the mandibular premolars in six beagle dogs. The defects were randomly treated as follows: open flap debridement (OFD) + CM (CM), OFD + xHyA (xHyA), OFD + xHyA + CM (xHyA/CM) and OFD alone (OFD). At 10 weeks, the animals were euthanized for histological evaluation.

Results

The newly formed bone areas in the xHyA (4.04±1.51 mm^2) and xHyA/CM (4.32±1.14 mm^2) groups were larger than those in the OFD (3.25±0.81 mm^2) and CM (3.31±2.26 mm^2) groups. The xHyA (6.25±1.45 mm) and xHyA/CM (6.40±1.35 mm) groups yielded statistically significantly ($P< 0.05$) greater formation of new connective tissue attachment (i.e., new cementum, with inserting connective tissue fibers) compared with the OFD (1.47±0.85 mm) group. No significant differences were observed in any of the histomorphometric parameters between the xHyA and xHyA/CM groups. Complete furcation closure was not observed in any of the four treatment modalities.

Conclusions

Within their limits, the present results suggest that the use of xHyA with or without CM positively influences periodontal wound healing in surgically-created, acute-type class Ⅲ furcation defects.

Keywords

hyaluronic acid, periodontal wound healing/regeneration, furcation defect, collagen matrix, biomaterials, animal study

Clinical relevance

Scientific rationale for study: The potential effect of xHyA with or without volume stable collagen matrix (CM) on periodontal wound healing/regeneration in class Ⅲ furcation defects is currently unknown.

Principal findings: The application of xHyA with or without CM promoted periodontal healing in class Ⅲ furcation defects, although complete furcation closure was not achieved.

Practical implications: The present findings indicate that xHyA is an effective biomodulator to facilitating periodontal wound healing/regeneration in class Ⅲ furcation defects. Further studies are needed to determine the optimal scaffold/carrier for xHyA to further improve the outcomes of regenerative surgery.

1 Introduction.

Periodontal regeneration is one of the ultimate goals of periodontal therapy because of the potential to change the prognosis of questionable or even hopeless teeth to one of retention and long-term maintenance (Cortellini et al., 2017, 2020; Stavropoulos et al., 2021), thus preventing tooth loss and saving dental care costs (Cortellini et al., 2017, 2020; Fardal & Grytten 2013). In recent decades, various periodontal reconstructive/regenerative procedures such as the use of bone substitutes, barrier membranes, various biologics/growth factors (e.g., enamel matrix derivative: [EMD], platelet-derived growth factor: [PDGF] and basic fibroblast growth factor: [FGF-2]) have been used alone or in various combinations. Positive clinical (including clinical attachment level [CAL] gain and probing pocket depth [PPD] reduction) and radiographical outcomes have been reported, together with high levels of patient satisfaction (Camelo et al., 1998; Kitamura et al., 2016; Li et al., 2017; Miron et al., 2016). Human histological evidence for true regeneration of root cementum, alveolar bone, and periodontal ligament has also been reported especially for decalcified freeze-dried bone allograft, demineralized bovine bone mineral, EMD and PDGF (Sculean et al., 2015; Nibali et al., 2020). Despite the successful results, several studies have demonstrated inconsistent and/or unfavorable results following various periodontal reconstructive/regenerative approaches, possibly due to factors such as the different nature of the biomaterials/biologics employed, the combinations used (Tu et al., 2010; Miron et al., 2016; Lee et al., 2017), and

post-operative complications (Sanz et al., 2004; Yoshinuma et al., 2012). Therefore, new biologics with excellent biocompatibility, easy handling, and lower initial cost need to be developed to further enhance periodontal wound healing/regeneration and to increase the predictability of clinical outcomes.

Hyaluronic acid (HyA), an anionic, non-sulfated glycosaminoglycan structured biomolecule, is a major natural component of the extracellular matrix in many tissues including the skin, joints, eyes, and periodontium (Eliezer et al., 2019; Pilloni et al., 2019). Physiochemically and biologically, HyA is hygroscopic (Dahiya & Kamal, 2013; Pilloni et al., 2019), viscoelastic (Dahiya & Kamal, 2013), bacteriostatic (Pimazar et al., 1999; Dahiya & Kamal, 2013; Eliezer et al., 2019), antibacterial (Eick et al., 2013), anti-inflammatory (Sasaki & Watanabe 1995; Moseley et al., 2002; Dahiya & Kamal, 2013; Eliezer et al., 2019), and anti-edematous (Dahiya & Kamal, 2013; Eliezer et al., 2019). Extensive in vitro studies have demonstrated that HyA significantly stimulates clot formation (Scully et al., 1995; Pilloni et al., 2019), induces angiogenesis (West et al., 1985; Pilloni et al., 2019), and increases osteogenesis (Pilloni & Bernard 1998; Pilloni et al., 2019). HyA is currently also available in cross-linked form (cross-linked HyA: xHyA) for various tissue engineering applications, serving as biologics/scaffolds to further improve the overall mechanical properties and provide a longer degradation period compared with non-cross-linked HyA (Takeda et al., 2011; Choi et al., 2015; Fujioka-Kobayashi et al., 2016). Very recent studies have shown that

the application of xHyA yielded statistically significant improvements characterized by PPD-reduction and CAL gain in human intrabony defects at 24 months (Pilloni et al., 2021), and effectively induced periodontal tissue regeneration in recession and 2-wall-intrabony defects in dogs (Shirakata et al., 2021a, 2021b).

It has been repeatedly demonstrated that the management of furcation involved molars is still one of the most challenging endeavors for the clinician. Due to their complicated anatomical characteristics (i.e., horizontal and vertical bone loss, presence of multiple roots with or without concavities and furrows, long trunks, etc.) furcation areas are hardly accessible for adequate instrumentation. Additionally, due to the wide avascular surfaces, coupled with the horizontal and vertical attachment and bone loss, it is extremely difficult to establish a biologic environment that facilitates periodontal wound healing/regeneration which in turn, may clinically lead to furcation closure (Bower, 1979; Waerhaug, 1980; Laugisch et al., 2019; Jepsen et al., 2020). These anatomical and biological considerations may explain the more unpredictable and heterogenous outcomes obtained following various regenerative approaches obtained in furcations compared to those in recession and intrabony defects (Sculean et al., 2008, 2015; Miron et al., 2016; Laugisch et al., 2019; Dommisch et al., 2020; Jepsen et al., 2020; Nibali et al. 2020). Obviously, novel treatment strategies leading to periodontal regeneration in furcation defects are stringently needed.

No data is, however, currently available on the potential effects of xHyA on periodontal wound healing/regeneration in furcation defects. In particular, class Ⅲ furcation defects in experimental animals are considered appropriate for evaluating the potential regenerative effects of biologics/biomaterials, although human class Ⅲ furcation lesions represent challenging defects that generally preclude periodontal regenerative therapy because of their unfavorable anatomical characteristics for clot and flap stabilization without sufficient surrounding periodontal tissues (Rossa et al., 2000; Roriz et al., 2006; Suiad et al., 2010; Laugisch et al. 2019). Therefore, the aim of this study was to histologically evaluate the effects of xHyA alone or in combination with a volume-stable collagen matrix (CM) on periodontal wound healing/regeneration in class Ⅲ furcation defects in dogs.

2 Materials and Methods

2.1 Animals

Six healthy male beagle dogs, 14–17 months of age and weighing 9.5–12.9 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, Ltd, Kagoshima, Japan. They were kept in individual cages at 20–26 ℃, with relative humidity of 30%–70 %, and a 12-hour light/dark cycle. Approximately 300 g of solid food (NVE-10, Nippon Pet Food, Co. Ltd, Tokyo, Japan) 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 (approval no. D20010; conducted from 28 May 2020 through18 October 2021). This study conformed to the ARRIVE guidelines for preclinical animal experiments.

2.2 Surgical protocol

All surgeries were performed by one experienced surgeon (Yo. S.) under general and local anesthesia using aseptic routines. Before the surgical interventions, antibiotics (dihydrostreptomycin sulfate aqueous suspension for injection, 0.05 ml/kg; Mycillin Sol Meiji for veterinary use, Meiji Seika Pharma Co. Ltd, Tokyo, Japan) were administered intramuscularly. General anesthesia was induced with intramuscular injection using medetomidine hydrochloride (Domitor®, 0.08 ml/kg IM; Orion Corporation, Espoo, Finland), 0.08 ml/kg of midazolam (Dormicum®, IM; Maruichi Pharmaceutical, Osaka, Japan) and 0.02 ml/kg of butorphanol tartrate (Vetorphale® 5 mg, Meiji Seika Pharma, Tokyo, Japan). After sedation, the anesthesia was maintained by inhalation of sevoflurane (0.5%–5.0%, Mylan Pharma Co., Ltd. Osaka, Japan) and a nitrogen/oxygen (2:1) mixture using an intracircuit vaporizer for spontaneous breathing. Local anesthesia (lidocaine HCl/epinephrine 2%, 1:80,000; Xylocaine; Fujisawa Inc., Osaka, Japan) was administrated to reduce peri-operative pain and bleeding. The mandibular second, third, and fourth premolars (P2, P3 and P4) (six sites per dog) were employed for experiments (**Figure 1a**). Following sulcular incisions and elevation of the mucoperiosteal flap, class Ⅲ furcation defects were created

surgically with the use of round burs and bone chisels with a sterile saline coolant (**Figure 1b**). The furcation defects were standardized with a millimeter probe and measured 5.0 mm in the occluso-apical direction and 5.0 mm in the mesio-distal direction. The bone between the mesial and distal roots was removed from the buccal and lingual sides. The exposed root surfaces were planed with Gracey curettes to remove the periodontal ligament and root cementum. Reference notches were made with a #1 round bur on the root surface at the base of the defects and on the crown surface, to indicate the precise center plane/extension of the class Ⅲ furcation defects and to allow for optimal histomorphometric analysis. The 36 bilateral mandibular class Ⅲ furcation defects randomly received one of the following treatments: cross-linked porcine collagen matrix (Fibro-Gide®, Geistlich Pharma AG, Wolhusen, Switzerland) alone (CM), cross-linked hyaluronic acid gel (Hyadent BG®, REGEDENT AG, Zurich, Switzerland) alone (xHyA), HA + CM (xHyA/CM), and open flap debridement (OFD) as a surgical control (**Figure 1c**). In the CM group, CM (5 × 5 × 6mm/defect) was soaked with sterile saline (0.2 ml/CM) before being applied to the defects (**Figure 1c**). The xHyA gel (0.2 ml/defect) was applied to the root surfaces, and the defects were filled up to the adjacent alveolar crest in the xHyA group (**Figure 1c**). In the xHyA/CM group, the CM was fully saturated with xHyA (0.2 ml/CM) instead of sterile saline and the constructs were allowed to rest for 10 min. The constructs were then placed in the defects with moderate pressure (**Figure 1c**). Maximum care was taken during surgery to prevent

spiling of xHyA to the other site on 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 Inc., Flagstaff, AZ, USA) slightly coronal to the cementoenamel junction (**Figure 1d**) to obtain a complete tension-free coverage of the furcation defects and the regenerative materials. Sutures were removed after 2 weeks.

2.3 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 Co. Ltd, Matsumoto, Japan) and an antibiotic (Mycillin Sol) were administered daily for 2 days. Plaque control was maintained with routine (three times a week) flushing of the oral cavity with 2% chlorhexidine gluconate solution for 10 weeks after surgery.

2.4 Histologic preparation

Ten 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, Ltd, Osaka, Japan), dehydrated, and embedded in paraffin. Serial 6-μm-thick sections were then

prepared along the mesiodistal plane and were stained with hematoxylin and eosin or with azan.

2.5 Histomorphometric analysis

All specimens were analyzed under a light microscope (BX51; Olympus Corp., Tokyo, Japan) equipped with a computerized image system (WinROOF2015; Mitani Corporation, Tokyo, Japan). For histomorphometric analysis, three sections approximately 90 μm apart were selected from the most central area of each class Ⅲ furcation 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 (**Figure 2**) were measured by a single experienced blinded examiner (T.I.). The following area measurements were taken (*in* mm^2): 1. bone defect area (BDA): area limited by the apical line (N_1-N_2) and the root surface in the furcation region; 2. non-filled area (NFA): portion of the BDA not filled with any tissue; 3. epithelial tissue area (ETA): portion of the BDA filled with epithelial tissue; 4. connective tissue area (CTA): portion of the BDA filled with connective tissue; and 5. new bone area (NBA): portion of the BDA filled with new bone. The following linear measurements were taken (*in mm*): 1. defect height (DH): vertical length between the apical end of the root notches (N; N_1, N_2) and the coronal end of the furcation (fornix); 2. defect width (DW): horizontal length between the apical end of the root notches (N; N_1, N_2); 3. length of the root surface (LRS): length of the root surface from the mesial

notch to the distal notch; 4. tissue-free defect length (TFL): portion of the LRS with the absence of any new tissue formation; 5. junctional epithelial length (JEL): total linear extensions of the root surface covered by epithelial tissue (JEL_1＋JEL_2); 6. connective tissue adhesion length (CTL): total linear extensions of the root surface covered by connective tissue without cementum (CTL_1＋CTL_2); 7. new cementum formation length (NCL): total linear extensions of the root surface covered by new cementum (NCL_1＋NCL_2); and 8. new connective tissue attachment formation length (NAL): total linear extensions of the root surface covered by new cementum adjacent to newly formed bone, with functionally oriented collagen fibers (NAL_1＋NAL_2).

2.6 Statistical analysis

The primary outcome of this study was the histomorphometric outcome in terms of NAL, measured for the four treatment groups at 10 weeks. However, due to the limited number of preclinical studies in dogs with a comparable design and primary outcome, no power analysis for sample size calculation could be performed. The animal was used as the statistical unit (N=6) for histomorphometric analysis. Histomorphometric recordings from the selected step serial sections from each defect were used to calculate the mean scores for each animal. A nonparametrical analysis of variance (Kruskal-Wallis test) was used to detect statistically significant differences between the different treatment groups. When the Kruskal-Wallis test was significant, Steel-Dwass post hoc test was performed for multiple comparisons. *P*-values

less than 0.05 were considered as statistically significant. These analyses were performed using statistical software (BellCurve for Excel, Social Survey Research Information Co. Ltd, Tokyo, Japan).

3 Results

3.1 Clinical observations

All animals tolerated the surgical procedures well, and healing after the reconstructive surgeries occurred uneventfully. No severe inflammation or swelling and dehiscence of the flaps were observed throughout the entire experimental period.

3.2 Descriptive histology

In the OFD group, downgrowth of gingival epithelium was more evident (**Figures 3a, 4a, b**) than in the xHyA (**Figures 3c, 4g**) and xHyA/CM (**Figures 3d, 4j**) groups. However, spontaneous bone formation including large bone marrow spaces occurred to some extent (**Figures 3a, 4a**). Cementum formation was restricted below the bone crest in most sites**.** In addition, new connective tissue attachment formation was minimal at the level of the apical notch (**Figures 3a, 4a, c**). In the CM group, the CM was considerably resorbed, however, bone formation occurred to varying degrees in the furcation defects (**Figures 3b, 4d**). In four defects, bone formation was observed extending along the distal space onto the mesial root surface and along the mesial space onto the distal root surface with a dimple in the center (**Figure 3b**). In the remaining five sites of the CM group, bone formation extended from the

host bone towards the coronal region of the defects (**Figure 4d**). New cellular/acellular cementum was seen, with or without collagen fibers oriented parallel to or detached from the root surfaces (**Figure 4e, f**). The formation of bone and cementum was apparent and comparable in the xHyA and xHyA /CM groups (**Figures 3, 4**). New bone was observed extending close to the fornix of the furcation in these groups, although two defects in the HA/CM group presented an incomplete or comparable bone filling similar to some sites observed in the CM group (**Figure 3b, d**). In most of the xHyA-applied (xHyA and xHyA/CM) sites, 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 (**Figures 3, 4**). New cellular/acellular cementum, with inserting collagen fibers running perpendicular to the root surfaces, was observed covering approximately 60% of the denuded root surface (**Figures 3c, d, 4g, j**). The highly vascularized and dense new periodontal ligament-like tissue that formed between the new cementum and new bone, maintained its width up to the coronal portion in the xHyA and xHyA /CM groups (**Figure 4h, i, k, l**). Complete defect resolution was not achieved in any of the defects and treatment modalities. Neither root resorption nor ankylosis was observed in any of the defects.

3.3 Histomorphometric analysis

The results of the histomorphometric analysis are shown in **Tables 1 and 2**. No statistically significant differences were detected among the groups in regard to the measurements for

BDA, NFA, ETA, CTA, NBA, DH, DW, LRS, and TFL. The JEL on the denuded root surface was statistically significantly longer in the OFD group than in the xHyA and xHyA/CM groups. The length of connective tissue (without cementum formation) was statistically significantly greater in the OFD group than in the xHyA and xHyA/CM groups. The amount of new cementum (NC) in the xHyA and xHyA/CM groups was statistically significantly greater than that in the OFD group. The xHyA and xHyA /CM groups yielded statistically significantly greater formation of new attachment (i.e., linear length of NC adjacent to newly formed bone, with functionally oriented collagen fibers) when compared with the OFD group. No significant differences in any of the histometric parameters were observed between the xHyA and xHyA /CM groups.

4 Discussion

To the best of our knowledge, the present study has for first time histologically evaluated the potential effects on periodontal wound healing/regeneration of xHyA with or without CM in class Ⅲ furcation defects in a larger animal model (i.e., in dogs). The most notable finding of this study was that sites treated with xHyA (i.e., either with xHyA alone or xHyA/CM) demonstrated statistically significantly greater amounts of new connective tissue attachment formation (i.e., NAL) than sites treated with OFD. Specifically, in the xHyA-treated sites, dense functionally oriented collagen fibers with many blood vessels were observed between the newly formed cementum and the newly formed bone. Additionally, more new bone was

observed in the xHyA (40.70±12.80 %) and xHyA/CM (44.70±8.7 %) groups than in the OFD (35.10±14.10 %) and CM (30.10±15.40 %) groups, although there were no statistically significant differences among the treatment groups. The rates of bone formation in the xHyA-treated groups were equal to or greater than those obtained in previous animal studies evaluating the histomorphometric analysis following the application of β-TCP (Jiang et al., 2010), bovine bone mineral matrix (Suiad et al., 2010), and EMD (Fernandes et al., 2005) with or without barrier membranes in chronic class Ⅲ furcation defects in dogs. Although these comparisons should be interpreted with caution due to the differences in acute vs. chronic defects, species (beagle or mongrel dog) and length of healing period (10 weeks vs. 12 weeks), the use of the synthetic product xHyA shows promise as a safe, simple and cost-effective alternative approach for supporting periodontal wound regeneration. Moreover, the present outcomes are in accordance with previous studies that show favorable periodontal regeneration or clinical improvements in PPD reduction and CAL gain following the same xHyA application in intrabony and gingival recession defects (Shirakata et al., 2021a, 2021b, Pilloni et al., 2021). These positive results may be explained by the numerous in vitro studies that have demonstrated that xHyA strongly sustains blood clot stability, attracts growth factors (Shimabukuro et al., 2005; Asparuhova et al., 2019; Pilloni et al., 2021), and increases angiogenesis (West et al., 1995; Pilloni et al., 2019) and osteogenesis (Pilloni & Bernard, 1998; Pilloni et al., 2019), while maintaining the fundamental physiochemical and biological

properties of HyA (Dahiya & Kamal, 2013). In particular, studies have reported that the interaction between CD44, a cell surface molecule with HyA in periodontal ligament (PDL) cells, is pivotal for the proliferation and migration of PDL cells (Shimabukuro et al., 2011; Al-Rekabi et al., 2019). HyA also induces early osteogenic differentiation of PDL cells (Fujioka-Kobayashi et al., 2017), increases the migratory and proliferative properties of gingival fibroblasts (Asparuhova et al., 2019), and maintains the stemness of mesenchymal stromal cells and pre-osteoblasts (Asparuhova et al., 2020) for accelerating the early phase of periodontal wound healing/regeneration.

The rationale for using a volume stable CM as a scaffold or carrier for xHyA was based on very recent data from in vitro experiments demonstrating that PDL and endothelial cells exhibited increased migration toward this matrix while, at the same time, the matrix facilitated the adsorption and long-term release (up to 12 days) of a number of key growth factors such as transforming growth factor (TGF)-β1, PDGF-BB, FGF-2, and growth differentiation factor (GDF)-5 responsible for periodontal regeneration (Asparuhova et al., 2021). Moreover, the stimulatory effect of the CM on the expression of antifibrinolytic genes was synergistically enhanced by TGF-β1, PDGF-BB, or FGF-2, whereas the strong inhibitory effect of the CM on the expression of profibrinolytic genes was reversed by PDGF-BB, FGF-2, or GDF-5 (Asparuhova et al., 2021). These in vitro findings were corroborated by the results of an animal study that histologically evaluated the effect of this CM on periodontal regeneration in

2-wall-intrabony defects (Imber et al., 2021a, 2021b). The histological analysis demonstrated statistically significantly higher formation of PDL, root cementum, and bone in defects treated with OFD + CM compared with those treated with OFD alone, thus providing evidence for the potential of the CM to facilitate periodontal regeneration (Imber et al., 2021a). Moreover, the immunohistochemical analysis revealed that the regenerated PDL demonstrated statistically significantly more blood vessels compared with the pristine PDL, thus suggesting a positive effect of the CM on angiogenesis (Imber et al., 2021b).

However, no statistically significant differences in any of the histomorphometric parameters were found between the groups treated with either xHyA and xHyA/CM groups. Additionally, no statistically significant differences were detected between the OFD and CM groups in any of the evaluated histomorphometric parameters, while the CM group demonstrated a large variation in healing pattern with or without periodontal regeneration. The findings indicate that the biologic effects triggered by xHyA might have masked the additional influence of CM on the regeneration process and the CM seemed to inhibit periodontal wound healing/regeneration in this experimental model. In this study, the CM was used in a commercially available standardized size (5 × 5 × 6mm) and tightly placed into the relatively narrow (approximately 3 mm) mesiodistal defect space between the mesial and distal roots to prevent collapse of the mucoperiosteal flap. However, the use of condensed CM with a relatively longer degradation period in this particular class Ⅲ furcation defects might

jeopardize the invasion of cells, nutrients, and vascular supply desirable for periodontal wound regeneration (Shirakata et al., 2002; Lee et al., 2012; Yoshinuma et al., 2012), and thus it cannot be ruled out the possibility that the used CM was not the optimal carrier/scaffold. At this point it is important to mention that at present no data are available on the binding and release kinetics data for the CM/xHyA construct. Therefore, further investigations are required to clarify the binding and release kinetics of xHyA when used in combination with CM. Additionally, the CM might not play a role of cell occlusive barrier membrane for ensuring marginal sealing and preventing migration of junctional epithelium and gingival connective tissue and/or plaque accumulation (Pontoriero et al., 1992; Hovey et al., 2006; Roriz et al., 2006; Jiang et al., 2010) in the furcation defects with extremely short length of root trunk and concavities (Lu 1992; Novaes et al., 2001) in dogs. Consequently, xHyA alone appears to possess the biological potential to positively influence periodontal wound healing compared to xHyA/CM without any additional effects of using CM in this study.

When interpreting the present findings, it should be kept in mind that, on the one hand, the positive outcomes following the use of xHyA were obtained in a relatively small number of animals and acute-type defects showing favorable spontaneous healing without strong bacterial challenges and inflammatory reactions (Sculean et al., 2008; Struillou et al., 2010; Donos et al., 2018), which may have limited the statistical power of the study. On the other

hand, the inclusion of a higher number of animals and defects is limited by obvious ethical and practical reasons.

The fact that complete closure of class Ⅲ furcation defects was not observed in any of the four treatment modalities is consistent with previous findings from preclinical and clinical studies that have clearly shown that these types of defects (regardless if an acute- or chronic-type) represent extremely challenging scenarios for periodontal regeneration (Rossa et al., 2000; Fernandes et al., 2005; Roriz et al., 2006; Jiang et al., 2010; Suiad et al., 2010; Laugisch et al., 2019). Further research should aim to develop novel strategies involving the use of xHyA or other biologics and their combinations with various types of carriers prior to clinical application in human class Ⅲ furcation defects.

5 Conclusion

In conclusion, within their limits, the present results suggest that the use of xHyA with or without CM positively influences periodontal wound healing in surgically-created, acute-type class Ⅲ furcation defects.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The experimental protocol was reviewed and approved by the ethical committee of the Animal Research Center of Kagoshima University, Japan (Approval No. D20010). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent

For this kind of study, formal consent is not required.

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Table 1 Histomorphometric area measurements for each treatment modality (mean ± SD in mm^2; N = 6 animals)

Parameters	Treatment group				Statistically significant differences
	(1) OFD	(2) CM	(3) xHyA	(4) xHyA/CM	
BDA	10.26±4.37	10.66±4.32	10.39±3.54	10.19±4.27	NS
NFA	0.31±0.27	0.50±0.58	0.49±0.83	0.56±0.71	NS
ETA	0.73±0.84	0.57±0.47	0.45±0.71	0.36±0.50	NS
CTA	2.88±3.25	3.91±2.61	1.72±1.23	1.84±1.77	NS
NBA	3.25±0.81	3.31±2.26	4.04±1.51	4.32±1.14	NS

NS, non-significant; BDA, bone defect area; NFA, non-filled area; ETA, epithelial tissue area; CTA, connective tissue area; NBA, new bone area

Table 2 Histomorphometric linear measurements for each treatment modality (mean ± SD in mm; N = 6 animals)

Parameters	Treatment group				Statistically significant differences
	(1) OFD	(2) CM	(3) xHyA	(4) xHyA/CM	
DH	4.65±0.99	4.90±1.08	4.90±0.98	4.83±1.15	NS
DW	2.90±0.75	2.97±0.66	2.80±0.64	2.81±0.78	NS
LRS	11.37±1.93	11.62±2.46	11.61±2.16	11.37±2.71	NS
TFL	1.13±1.03	1.21±1.40	1.00±0.57	1.23±0.68	NS
JEL	3.07±1.28	3.72±2.44	0.90±0.64	0.76±0.53	1 vs 3* 1 vs 4*
CTL	4.63±1.61	1.85±1.53	1.13±0.76	1.33±1.03	1 vs 3* 1 vs 4*
NCL	2.54±1.35	4.45±2.65	7.47±1.85	6.86±1.48	1 vs 3* 1 vs 4*
NAL	1.47±0.85	3.40±2.46	6.25±1.45	6.40±1.35	1 vs 3* 1 vs 4*

NS, non-significant; * statistically significant differences ($P < 0.05$)

OFD, open flap debridement; CM, collagen matrix; xHyA, cross-linked hyaluronic acid gel; DH, defect height; DW, defect width; LRS, length of the root surface; TFL, tissue-free defect length; JEL, junctional epithelium length; CTL, connective tissue adhesion (without cementum) length; NCL, new cementum formation length; NAL, new connective tissue attachment length

Figure legends

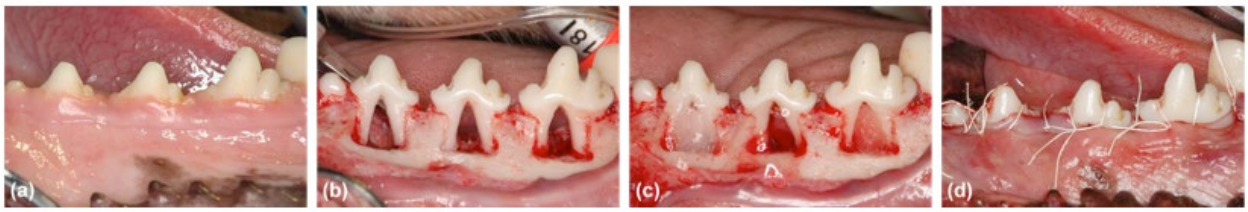

Figure 1.

Clinical illustration of the treatment procedure. (a) Prior to reconstructive surgery. (b) Surgically created class Ⅲ furcation defects. (c) The defects received cross-linked hyaluronic acid gel (xHyA) with collagen matrix (CM) (xHyA/CM), xHyA and CM (left to right). (d) Flap repositioning and suturing.

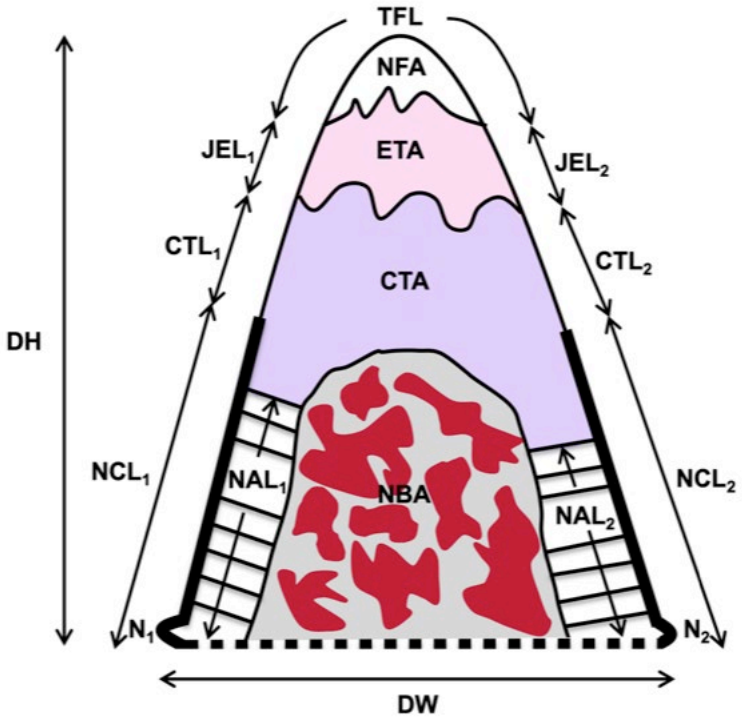

Figure 2

Landmarks/parameters used in histomorphometric analysis. DH, defect height, DW, defect width, NFA, none-filled area; ETA, epithelial tissue area; CTA, connective tissue area; NBA, new bone area; LRS, length of the root surface (TFL+JEL+CTL+NCL); TFL, tissue-free defect length; JEL, junctional epithelial length; CTL, connective tissue adhesion length; NCL, new cementum formation length; NAL, new connective tissue attachment length; N (N_1, N_2), apical notch

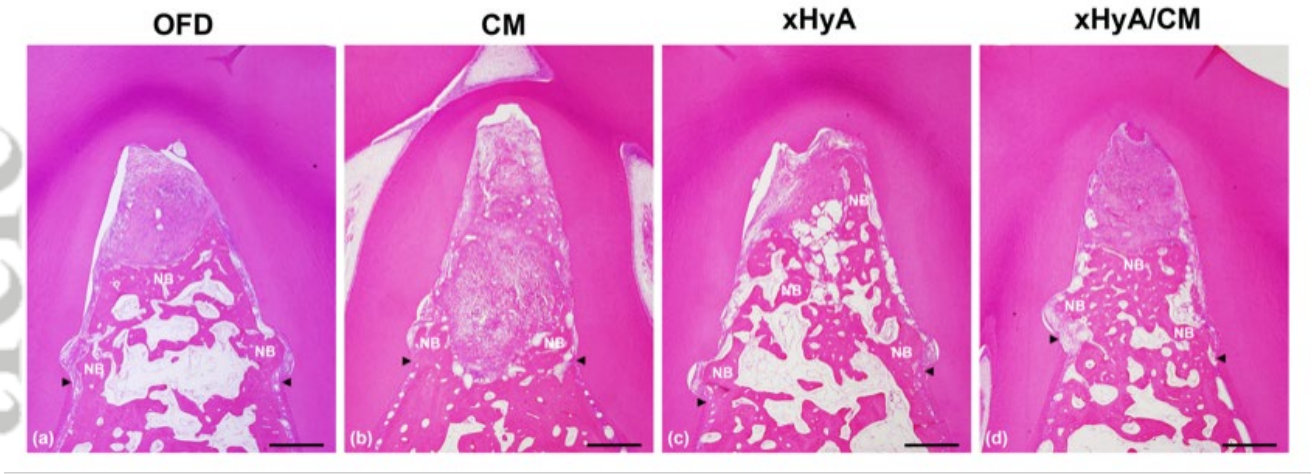

Figure 3. Representative photomicrographs of class Ⅲ furcation defects in different groups. Open flap debridement (OFD) group. **(a)** Histologic overview of mandibular right fourth premolar. Collagen matrix (CM) group. **(b)** Histologic overview of mandibular left third premolar. Cross-linked hyaluronic acid (xHyA) group. **(c)** Histologic overview of mandibular left fourth premolar. Cross-linked hyaluronic acid with collagen matrix (xHyA/CM) group. **(d)** Histologic overview of mandibular right second premolar. (scale bar, 1 mm; hematoxylin and eosin staining). Arrowhead: notch (apical extension of root planing)**;** NB, new bone

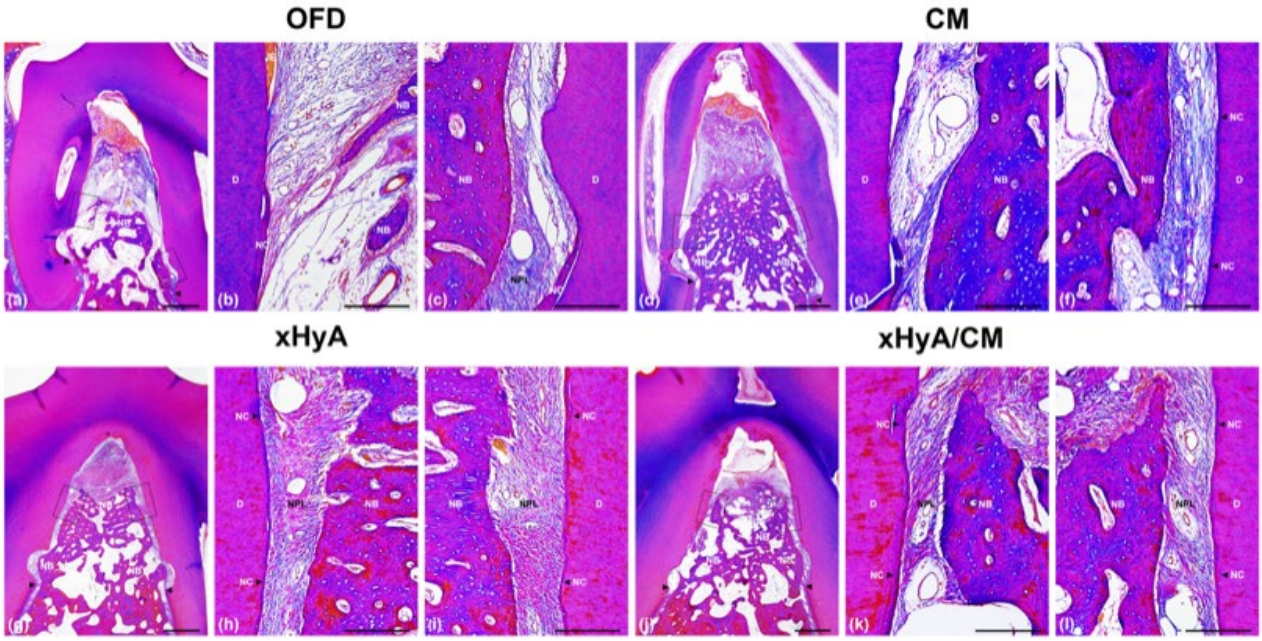

Figure 4. Representative photomicrographs of class Ⅲ furcation defects in different groups. Open flap debridement (OFD) group. **(a)** Histologic overview of mandibular right second premolar (scale bar, 1 mm; azan staining). Arrowhead: notch (apical extension of root planing). **(b)** Higher magnification of the framed area (left) in Figure 4a (scale bar, 200 μm; azan staining). **(c)** Higher magnification of the framed area (right) in Figure 4a (scale bar, 200 μm; azan staining). Collagen matrix (CM) group. **(d)** Histologic overview of mandibular right second premolar (scale bar, 1 mm; azan staining). Arrowhead: notch (apical extension of root planing). **(e)** Higher magnification of the framed area (left) in Figure 4d (scale bar, 200 μm; azan staining). **(f)** Higher magnification of the framed area (right) in Figure 4d (scale bar, 200 μm; azan staining). Cross-linked hyaluronic acid (xHyA) group. **(g)** Histologic overview of mandibular left second premolar (scale bar, 1 mm; azan staining). **(h)** Higher magnification

of the framed area (left) in Figure 4g (scale bar, 200 μm; azan staining). **(i)** Higher magnification of the framed area (right) in Figure 4g (scale bar, 200 μm; azan staining). Cross-linked hyaluronic acid with collagen matrix (xHyA/CM) group. **(j)** Histologic overview of mandibular left third premolar (scale bar, 1 mm; azan staining). **(k)** Higher magnification of the framed area (left) in Figure 4j (scale bar, 200 μm; azan staining). **(l)** Higher magnification of the framed area (right) in Figure 4j (scale bar, 200 μm; azan staining). D, root dentin; JE, apical end of junctional epithelium; NB, new bone; NC, new cementum; NPL, new periodontal ligament