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APPLICATION OF A BMP2-BINDING HEPARAN SULPHATE TO PROMOTE PERIODONTAL REGENERATION

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Abstract

Periodontitis is the most common inflammatory disease that leads to periodontal defects and tooth loss. Regeneration of alveolar bone and soft tissue in periodontal defects is highly desirable but remains challenging. A heparan sulphate variant (HS3) with enhanced affinity for bone morphogenetic protein-2 (BMP2) that, when combined with collagen or ceramic biomaterials, enhances bone tissue regeneration in the axial and cranial skeleton in several animal models was reported previously. In the current study, establishing the efficacy of a collagen/HS3 device for the regeneration of alveolar bone and the adjacent periodontal apparatus and related structures was sought. Collagen sponges loaded with phosphate-buffered saline, HS3, BMP2, or HS3 + BMP2 were implanted into surgically-created intra-bony periodontal defects in rat maxillae. At the 6 week end-point the maxillae were decalcified, and the extent of tissue regeneration determined by histomorphometrical analysis. The combination of collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 resulted in a three to four-fold increase in bone regeneration and up to a 1.5 × improvement in functional ligament restoration compared to collagen alone. Moreover, the combination of collagen/HS3 + BMP2 improved the alveolar bone height and reduced the amount of epithelial growth in the apical direction. The implantation of a collagen/HS3 combination device enhanced the regeneration of alveolar bone and associated periodontal tissues at amounts comparable to collagen in combination with the osteogenic factor BMP2. This study highlights the efficacy of a collagen/HS3 combination device for periodontal regeneration that warrants further development as a point-of-care treatment for periodontitis-related bone and soft tissue loss.

Keywords: Heparan sulphate, glycosaminoglycan, periodontal regeneration, bone tissue, alveolar bone, tooth, growth factor, bone morphogenetic protein-2.

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List of Abbreviations

%TL	relative tooth length
BA	bone area
BMP2	bone morphogenetic protein-2
CEJ	cementoenamel junction
Col	collagen
DA	defect area
DL	defect length
EDTA	ethylenediaminetetraacetic acid
EMD	enamel matrix protein derivative
FGF2	fibroblast growth factor-2
GDF5	growth and differentiation factor-5
GTR	guided tissue regeneration
H&E	haematoxylin and eosin
HS3	BMP2-binding HS
HS	heparan sulphate
IGF	insulin growth factor
PBS	phosphate-buffered saline
PDGFbb	platelet-derived growth factor beta polypeptide b
PDL	periodontal ligament
SD	standard deviation
TL	tooth length
VEGF	vascular endothelial growth factor

Introduction

Periodontitis, an inflammatory disease affecting the supporting tissues of teeth, is the most common dental disease in humans, with 11 % of the global population or 743 million people estimated to have severe periodontitis in 2010 (Papapanou and Susin, 2017). In the United States alone, over 47 % of the adult population has periodontitis (Eke *et al.*, 2012). The main cause of inflammation that leads to periodontitis is the accumulation of bacteria on the teeth, also known as dental plaque. Bacterial accumulation rarely causes infections, but the inflammatory response to these bacteria may contribute to a progressive destruction of collagenous and bony tissues around the teeth that, if left untreated, may lead to the loosening and premature loss of teeth (Loesche and Grossman, 2001). As a result, the patient's oral health, nutritional intake and general well-being would be greatly affected. In addition, the inflammatory responses triggered by periodontitis have been reported to exacerbate other systemic disorders, such as diabetes, coronary artery disease and stroke (Liu *et al.*, 2008).

Current clinical treatment of periodontitis consists mainly of scaling, root planing and open-flap debridement to remove bacterial accumulation and to prevent further inflammation and disease progression (Deas *et al.*, 2016). Although the disease progression is arrested and clinical symptoms are eradicated, these treatments often do not restore the damaged tissues (Cortellini *et al.*, 2007). Achieving regeneration of the periodontal apparatus is challenging as it involves sequential and special reconstruction of three separate tissues – the PDL, cementum and alveolar bone, all of which have very limited regenerative potential (Chen and Jin, 2010). Without intervention, the periodontal defects would often be filled with fibrous and epithelial tissues (Villar and Cochran, 2010), preventing bone and PDL from refilling the pocket. This leads to persistence of a residual periodontal pocket and higher risk of disease progression such as bone resorption and poor prognosis of the tooth (Chen and Jin, 2010; Sculean *et al.*, 2015).

Some tissue engineering approaches have been developed to restore periodontal tissues. One common approach is GTR, which uses a physical barrier to cover the defect with the aim of preventing epithelial infiltration and allowing osteogenic regeneration within the defect (Lin *et al.*, 2010). While GTR is generally successful in restoring maxillofacial and calvarial bone defects, its success in regenerating lateral and vertical periodontal tissues is highly variable (Retzepi and Donos, 2010). In addition, this approach involves technically difficult procedures and potential complications such as opening of the barrier and bacterial infection (Iwata *et al.*, 2014). Other approaches use bone grafts in periodontal defects to promote bone formation and periodontal regeneration, and a wide range of bone grafting materials have been applied for this purpose (Reynolds *et al.*, 2010). While bone grafts lead to some success in the regeneration of alveolar bone, they do not always regenerate PDL, cementum and tissue interfaces in true periodontal tissue, and may lead to root resorption and ankylosis.

The microenvironment of the periodontal defect is a crucial variable that could be engineered to promote the survival, proliferation and differentiation of preferred cell types (Lee *et al.*, 2010). As wound healing and tissue regeneration are driven by numerous signalling molecules and cytokines within a well-defined microenvironment, one of the primary strategies of periodontal tissue engineering is to provide growth factors to simulate an artificial environment for cell homing and tissue regeneration induction (Kao *et al.*, 2009). Since periodontal regeneration is a multicellular process involving a complex network of biological mediators, an addition of supportive mediators would promote appropriate cellular proliferation and expression, and also catalyse the healing process of multiple tissues in a sequential order (Chen and Jin, 2010). For example, biological mediators involved in periodontal healing include FGF-2 (Murakami, 2011), IGF (Chen *et al.*, 2006), BMP2 (Miyaji *et al.*, 2010), GDF5 (Kwon *et al.*, 2010), and EMD (Esposito *et al.*, 2009; Francetti *et al.*, 2005).

In addition, glycosaminoglycans such as heparin and HS and their derivatives, which are known for their ability to bind various growth factors, are increasingly used as carriers for these factors (Hachim *et al.*, 2019). The utility of a BMP2-binding HS (HS3), as an adjuvant or as part of the scaffolding material in several *in vitro* and *in vivo* models for bone regeneration was demonstrated in previous studies (Bhakta *et al.*, 2018; Le *et al.*, 2019; Murali *et al.*, 2013; Quang Le *et al.*, 2020; Rai *et al.*, 2015; Smith *et al.*, 2018). In particular, HS3 was used without the addition of exogenous BMP2 that resulted in enhanced bone regeneration (Le *et al.*, 2019; Murali *et al.*, 2013; Rai *et al.*, 2015). It was suggested that HS3 may mediate the effect of endogenous growth factors like BMP2, which is secreted as part of the normal bone healing response. Through binding to endogenous growth factors like BMP2, HS3 could sequester the growth factor, protect it from enzymatic degradation and increase its activity (Murali *et al.*, 2013), thereby obviating the need for exogenous application of growth factors like BMP2.

It was shown previously that a combination device consisting of collagen/HS3 is able to generate an osteostimulatory response in long-bone defects, resulting in enhanced bone formation (Murali *et al.*, 2013). In the current study, it was reasoned that a similar response could be generated in alveolar bone defects and provide a viable alternative to bone grafting strategies for dental use. It was hypothesised that collagen/HS3 would provide a

pro-healing environment and enhance alveolar bone regeneration and periodontal tissue without the need for exogenous application of growth factors such as BMP2. Utilising an established periodontal defect animal model (Yu *et al.*, 2013), the bone healing efficacy and periodontal tissue restoration between collagen alone and collagen in combination with HS3, BMP2 or HS3+BMP2 was compared.

Materials and Methods

Materials

All reagents and chemicals were obtained from Sigma-Aldrich (USA) unless otherwise stated. HS3 was isolated from a crude HS mixture (Cat. # HO-03103, lot# HO-10697, Celsus Laboratories, Cincinnati, OH, USA) according to methods described previously (Murali *et al.*, 2013). Recombinant human bone morphogenetic protein-2 (BMP2) was obtained from the Infuse Bone Graph kit (Medtronic, Minneapolis, MN, USA). Zimmer CollaPlug Absorbable Collagen Wound Dressing was purchased from Zimmer Biomet, Warsaw, IN, USA.

Implant preparation

All steps were performed under aseptic conditions. Collagen scaffolds measuring 3 × 3 × 3 mm^3 were cut from the CollaPlug cylinders. Each scaffold was loaded with 10 µL of PBS solution containing the treatments. The treatment groups were: (1) collagen alone, $n = 4$; (2) 1 µg of BMP2, $n = 4$; (3) 5 µg of HS3, $n = 6$; and (4) 1 µg of BMP2 + 5 µg of HS3, $n = 6$. The collagen scaffolds were then lyophilised and kept at room temperature until implantation into the animals (no longer than two weeks from the preparation date). A Power analysis was performed (G*Power, Faul *et al.*, 2009) to determine the appropriate sample size needed to achieve statistical significance. Using a 40 % improvement in bone formation between treatment and control as the primary variable data (Cai *et al.*, 2018; Oortgiesen *et al.*, 2013; Yan *et al.*, 2015), a sample size of 6 per treatment group was recommended.

In vivo procedure

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of SingHealth under protocol 2014/SHS/1007 and were conducted in accordance with the national guidelines for the use of laboratory animals. In this study, the efficacy of the loaded scaffolds was evaluated with 11 adult Wistar rats (male, 8 weeks old, average weight 300 g) using the periodontal defect model previously described by Yu *et al.* (2013) with slight modifications. Two defects were created in each rat and the treatment was allocated randomly across the defects (Table 1). As the same model was being used in a parallel but unrelated study, one of the two defects in rats 9, 15, 17, and 18 were utilised for either experiment in accordance with animal reduction principles and ethics (Table 1).

Bilateral intra-bony three-wall defects were created by making a 3 mm full thickness incision along the alveolar ridge mesially to both maxillary first molars while the rats were under general anaesthesia through intubation (Fig. 1**a**,**b**). After the mucogingival flaps were lifted to expose the underlying bone and root surface (Fig. 1**c**), part of the alveolar bone, root cementum and periodontal ligament were removed using a piezoelectric device (Piezosurgery®, Mectron, Carasco, Italy) loaded with OT5 B-tip (Ø 1.7 mm) to generate a defect with dimensions of 2 × 2 × 2 mm^3 (Fig. 1**d**). The defects were then rinsed with sterile saline, dried with sterile gauze and filled with the prepared collagen-scaffold

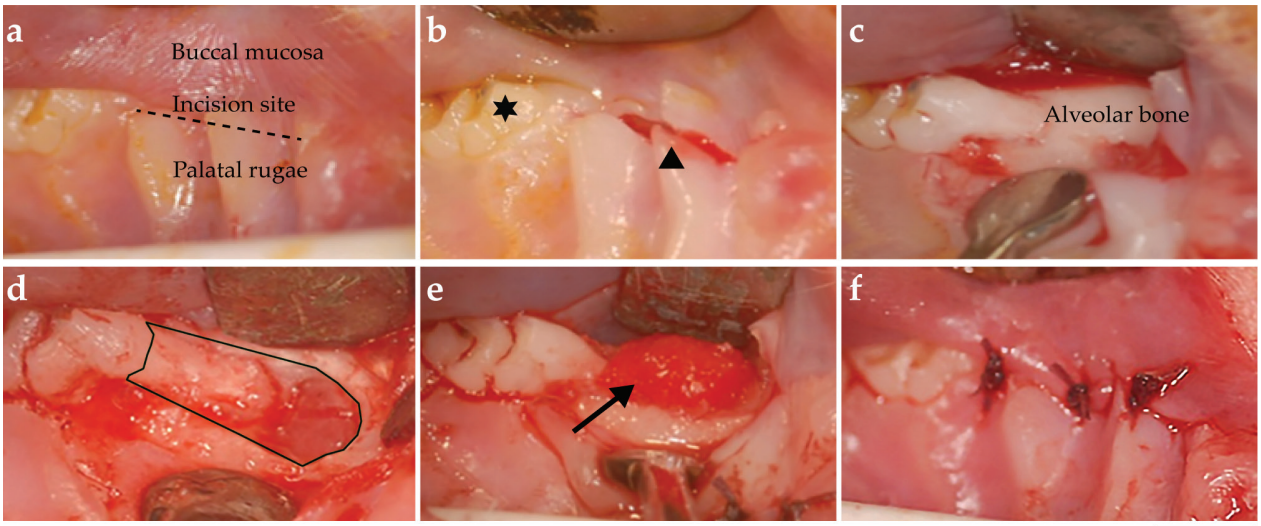

Fig. 1. Surgical procedures. (**a**) Surgical site at edentulous alveolar ridge at the mesial surface of maxillary first molar. (**b**) Site of incision. (**c**) Flap raised and alveolar crest bone exposed. (**d**) Intrabony defect. (**e**) Implants were inserted in the defect area. (**f**) Surgical site was closed with sutures. Star indicates upper first molar; triangle indicates incision site; arrow indicates the inserted collagen sponge; black demarcated line indicates defect site. Scale bar = 1 mm.

implants (Fig. 1**e**). The defects were then sealed by repositioning the mucogingival flaps and securing with resorbable sutures (Vicryl® 6-0, Ethicon Inc, Raritan, NJ, USA) (Fig. 1**f**). Post-operative analgesia was managed by subcutaneous injection of carprofen (Rimadyl, 5 mg/kg) once daily for 3 d.

Histology and histomorphometrical analysis

Six weeks after the implantation, the rats were sacrificed by increasing CO_2 concentration. The complete maxillae were harvested and excess tissue trimmed. The samples were fixed in 10 % formalin for 2 d and decalcified in 4 % EDTA for 3 weeks at room temperature. The maxillae were dissected into two halves through the palatial midline. After dehydration in graded series of ethanol and xylene, the samples were embedded in paraffin wax and cut with a microtome (Leica RM2165, Germany) into 5 μm sections in a mesiodistal plane. Histosections were obtained at approximately three equal levels across the whole width (2 mm) of the defect. The sections were stained with H&E for bone analysis and Ralis-modified tetrachrome for epithelial tissue and ligament observation. The slides were scanned using a 20× objective by a Metafer Slidescanner (Metasystems, Germany), exported to TIFF files and analysed using Photoshop (Adobe, USA). At least three separate sections were used for all analyses (Table 1). Histomorphometrical methods for the new bone area, alveolar crest gap, and functional ligament and epithelial growth in the apical direction were similar to a previous study (Yu *et al*., 2013), and is summarised in Fig. 2.

Statistical analysis

The obtained data are shown in Table 2. The value of each sample represents the mean of the analysed sections. Statistical analyses were performed by Student's *t*-tests (GraphPad Prism v7). A *p*-value < 0.05 was considered significant; * depicts $p \leq 0.05$, ** depicts $p \leq 0.01$ and *** depicts $p \leq 0.001$.

Results

General observations

One rat (R18) died prematurely due to anaesthesia failure, reducing the number of replicates in the control group to $n = 3$ (Table 1). One defect (R3-L) was created too deeply, penetrating into the left salivary gland, affecting healing of the periodontal region. This defect was excluded from the study, reducing the number of replicates in the HS3 group to $n = 5$ (Table 1). All other animals recovered well and gained weight throughout the 6-week observation period. At the time of sacrifice, the soft tissue around the surgical site healed without visible differences between the groups. Histologically, most samples exhibited no inflammation while a few samples showed minor inflammation that was restricted to the marginal region of the gingiva and did not affect the periosteum.

Table 1. Animal usage table outlining the treatment regimen and the number of histology sections used for analysis of each sample. NP: analysis not performed. * Protocol violation, sample removed from all analysis. ** Animal died during surgery.

				Number of histology sections used for analysis			
Animal ID	**Side**	**Sample ID**	**Treatment**	**New bone**	**Gap fraction**	**Functional ligament**	**Epithelial growth in apical direction**
Rat 1	Left	R1-L	Col alone	3	3	4	4
	Right	R1-R	Col/BMP2	3	3	4	4
Rat 2	Left	R2-L	Col/BMP2	3	3	4	4
	Right	R2-R	Col/HS3	3	3	3	3
Rat 3	Left	R3-L*	Col/HS3	NP	NP	NP	NP
	Right	R3-R	Col/HS3 + BMP2	3	3	4	4
Rat 4	Left	R4-L	Col/HS3 + BMP2	3	3	4	4
	Right	R4-R	Col/BMP2	3	3	4	4
Rat 5	Left	R5-L	Col/BMP2	4	3	4	4
	Right	R5-R	Col/HS3	3	3	4	4
Rat 6	Left	R6-L	Col/HS3	3	3	4	4
	Right	R6-R	Col/HS3 + BMP2	3	3	4	4
Rat 7	Left	R7-L	Col/HS3 + BMP2	3	3	4	4
	Right	R7-R	Col/HS3	3	3	4	4
Rat 8	Left	R8-L	Col/HS3	3	3	4	4
	Right	R8-R	Col/HS3 + BMP2	3	3	4	4
Rat 9	Left	R9-L	Col/HS3 + BMP2	3	3	4	4
Rat 15	Right	R15-R	Col alone	3	4	4	4
Rat 17	Left	R17-L	Col alone	3	4	4	4
Rat 18**	Right	R18-R	Col alone	NP	NP	NP	NP

Regeneration of the alveolar bone

In this periodontal model, a fixed volume of alveolar bone was removed together with the associated ligament and tooth root cementum to form a defect. To completely regenerate the periodontium, the alveolar bone must first recover to provide support for ligament attachment. Of the four treatment groups, collagen alone resulted in the least amount of new bone formation (19.6 ± 8.2 % BA/DA) (Fig. 3**a**; Table 2,3). With addition of HS3 (5 μg) to collagen, new bone formation significantly increased by ~ three-fold (59.9 ± 25.6 % BA/DA; p = 0.021*) compared to collagen alone. Also, 60 % of the defects treated with collagen/HS3 had % BA/DA outcomes higher than 66.8 %. In comparison, collagen alone failed to achieve a value higher than 33 % (Fig. 3**a**; Table 3). The addition of BMP2 (1 μg) to collagen also significantly increased new bone formation by ~ 4× (78.3 ± 7.1 % BA/DA; p < 0.001***) compared to collagen alone. Moreover, 100 % of the healing responses had % BA/DA values higher than 66.8 % (Fig. 3**a**; Table 3). There was no statistical difference in the amount of new bone formation (% BA/DA) between collagen/HS3 and collagen/BMP2 (p = 0.212). Similar to collagen/HS3 and collagen/BMP2, treatment with the combination of collagen/HS3 + BMP2 significantly increased the amount of new bone formation (80.35 ± 6.0 % BA/DA; p < 0.001***) compared to collagen alone. Notably, there was no statistical difference between treatments with collagen/HS3 + BMP2 and collagen/BMP2 (p = 0.6256) or collagen/HS3 (p = 0.088) (Fig. 3**a**; Table 2).

Representative images of H&E stained sections taken from treatment values approximating the median % BA/DA for each group are shown in Fig. 3**b**. From the histological sections, the defect areas could be identified by the interruption of hard tissue between the mesial root surface of the maxillary first

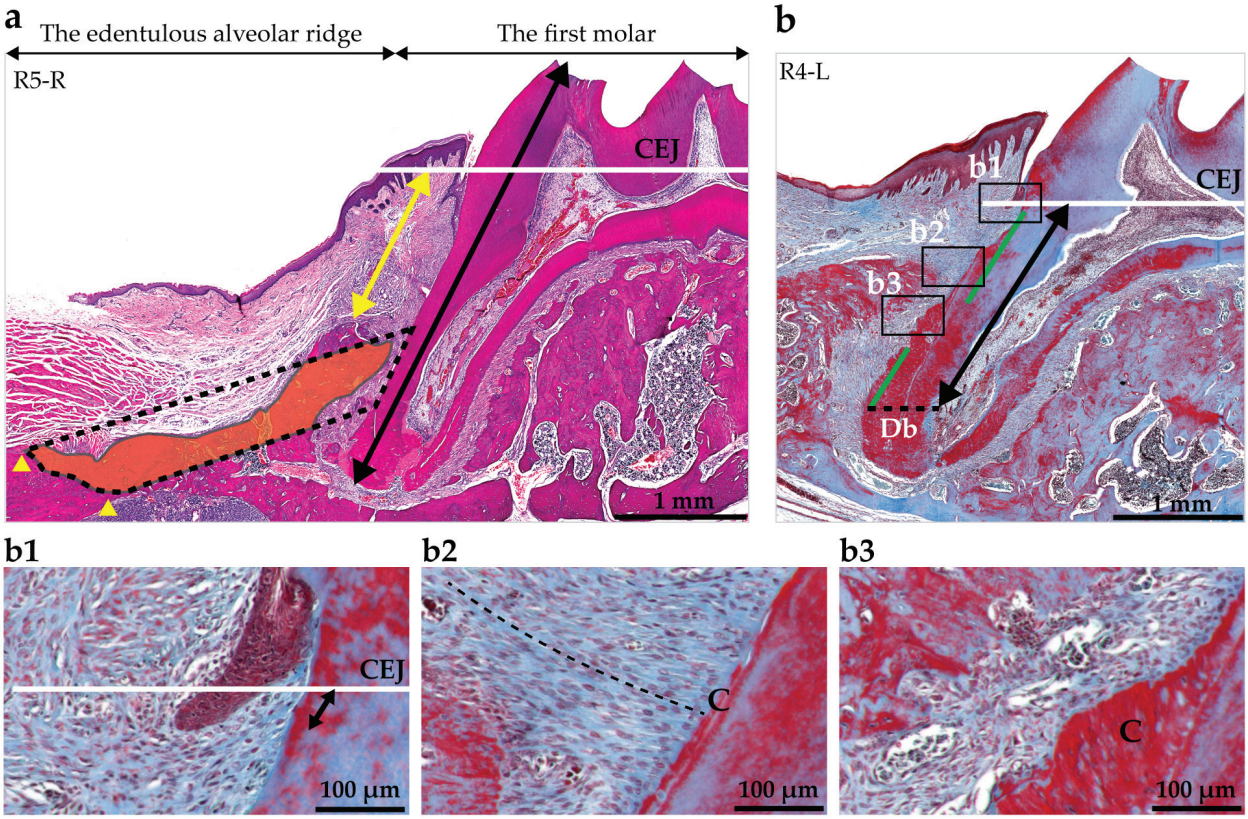

Fig. 2. Histomorphometrical analysis method. (**a**) Bone histomorphometry and gap fraction analysis was performed on H&E stained sections, in which bone, cementum, dentine and enamel were stained with varying shades of pink. For bone histomorphometry, the dashed line marks the region of interest (defect area) which is defined base on the surgical defect margins (yellow triangle); shaded area indicates the newly formed bone within the defect area. For gap-fraction analysis, the yellow line indicates the gap between the regenerated alveolar crest to the CEJ; black line indicates the tooth length (cusp to apical tip). (**b**) Functional ligament and epithelial apical growth analysis was performed on Ralis modified tetrachrome stained sections, in which ligament and collagen fibre was stained pale blue and epithelium was stained dark red. The green line marks regions of functional ligament; black line indicates the defect length, which is defined as the distance projected between the apical defect margin (Defect bottom – Db) and the CEJ. Scale bar = 1 mm. (**b1**) High magnification view showing the extent of the epithelial growth in apical direction. The black line indicates the length of epithelial growth below the CEJ. (**b2**) A region of functional ligament; the dashed line illustrates the ligament's angle of attachment to the cementum (C). Ligament bundles with ≥ 60° angle attachment to the cementum were considered functional. (**b3**) A region of non-inserted collagen bundles or bundles with < 60° attachment to the cementum (non-functional ligament). Scale bar = 100 μm.

Table 2. Summary of histomorphometrical data. #: Protocol violation, sample removed from all analysis; NP: analysis not performed. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Treatment	Sample ID	BA/DA (%)	Gap fraction (% TL)	Functional ligament (% DL)	Epithelial growth in apical direction (% DL)
Col alone	R1-L	28.3	16.6	38.1	16.3
	R15-R	18.3	24.4	60.6	8.1
	R17-L	12.2	39.2	59.9	16.7
	Mean	19.6	26.7	52.9	13.7
	SD	8.2	11.5	12.8	4.9
Col/HS3	R2-R	86.0	10.0	75.2	10.4
	R3-L	NP	NP	NP	NP
	R5-R	68.7	35.7	57.1	6.5
	R6-L	44.0	30.3	62.6	19.8
	R7-R	77.2	27.8	74.2	1.1
	R8-L	23.7	22.8	72.8	4.4
	Mean	59.9	25.3	68.4	8.4
	SD	25.6	9.8	8.1	7.2
	p (*vs.* Col alone)	0.021*	0.430	0.038*	0.155
Col/BMP2	R1-R	82.5	15.5	74.9	13.5
	R2-L	78.7	15.6	78.1	3.4
	R4-R	68.1	25.8	67.3	29.0
	R5-L	83.7	19.9	90.9	0.6
	Mean	78.3	19.2	77.8	11.6
	SD	7.1	4.9	9.8	12.8
	p (*vs.* Col alone)	< 0.001***	0.142	0.016*	0.402
	p (*vs.* Col/HS3)	0.212	0.293	0.158	0.650
Col/HS3 + BMP2	R3-R	86.8	21.0	73.1	3.3
	R4-L	80.9	22.2	77.7	6.0
	R6-R	77.7	13.3	80.5	6.3
	R7-L	87.0	9.2	67.9	6.2
	R8-R	71.2	16.0	63.7	14.0
	R9-L	78.5	13.3	68.9	2.3
	Mean	80.4	15.8	72.0	6.3
	SD	6.0	5.0	6.3	4.1
	p (*vs.* Col alone)	< 0.001***	0.040*	0.009**	0.024*
	p (*vs.* Col/HS3)	0.088	0.066	0.430	0.559
	p (*vs.* Col/BMP2)	0.626	0.326	0.282	0.365

Table 3. Summary of the number of data points in each shading zone in Fig. 3a and descriptive statistics of the BA/DA data.

	Bone area/Defect area (%)			
	Col	Col/HS3	Col/BMP2	Col/HS3 + BMP2
Shading zone				
66.8-100	0 % (0/3)	60 % (3/5)	100 % (4/4)	100 % (6/6)
33.4-66.7	0 % (0/3)	20 % (1/5)	0 % (0/4)	0 % (0/6)
0-33.3	100 % (3/3)	20 % (1/5)	0 % (0/4)	0 % (0/6)
Minimum	12.2	23.7	68.1	71.2
Maximum	28.3	86.0	83.7	87.0
Mean	19.6	59.9	78.3	80.4
Median	18.3	68.7	80.6	80.0
SD	8.2	25.6	7.1	6.0
Coefficient of variation	41.8	42.7	9.0	7.5

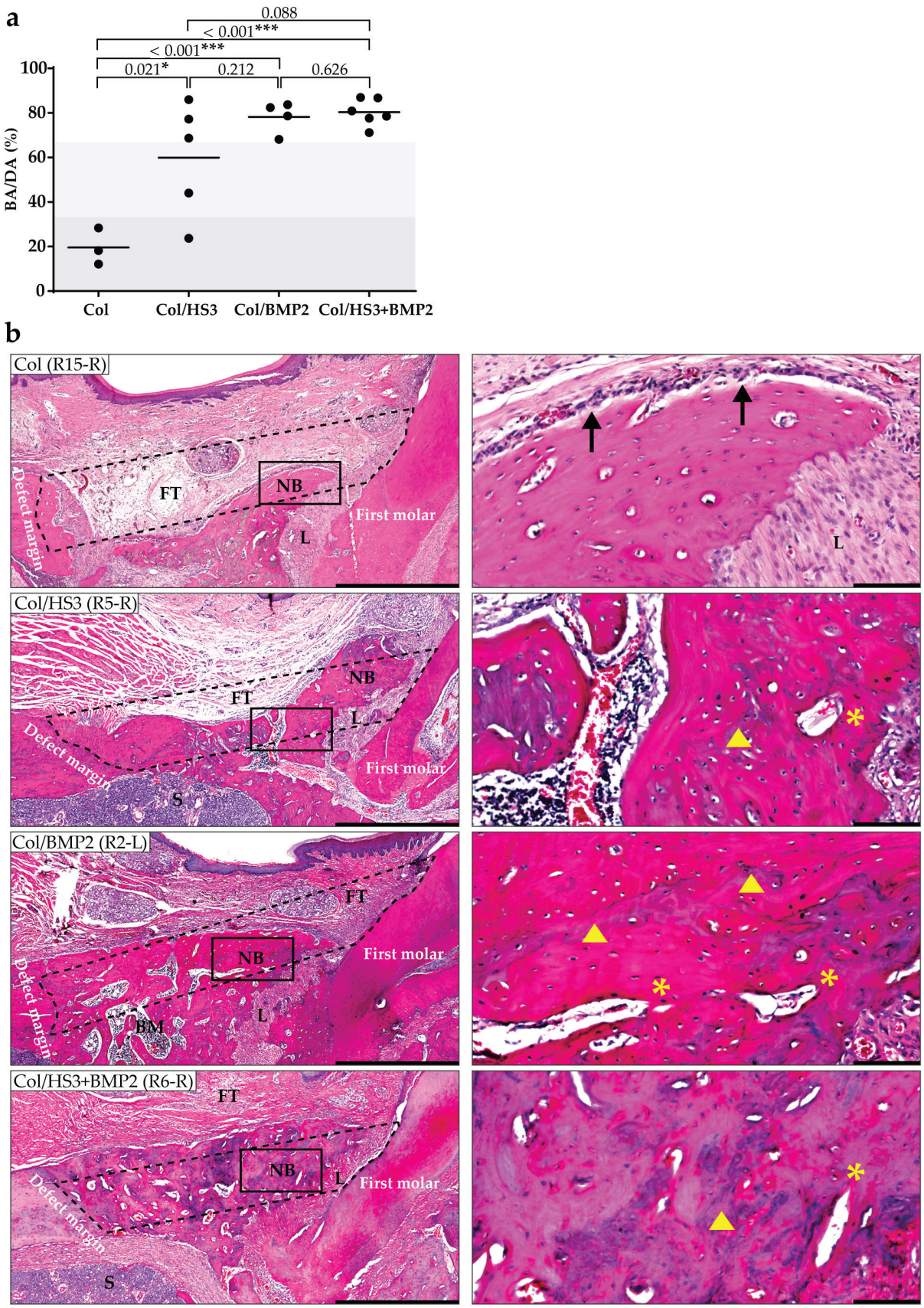

Fig. 3. New bone formation. (**a**) Graph illustrating new bone formation within defect area. The shading represents three equal ranges of BA/DA value (0 to 33.3; 33.4-66.7 and 66.8-100). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. (**b**) Representative H&E-stained histology images from each treatment group. Left column: overview (scale bar = 1 mm). Right column: high magnification (scale bar = 100 μm) view of area in the black box. The dashed line marks the defect area between the defect margin and root surface of the first molar. NB: new bone, BM: bone marrow, FT: fibrous tissue, L: ligament, S: salivary gland, black arrow: active osteoblast, yellow asterisk: Haversian canal, yellow triangle: cement line.

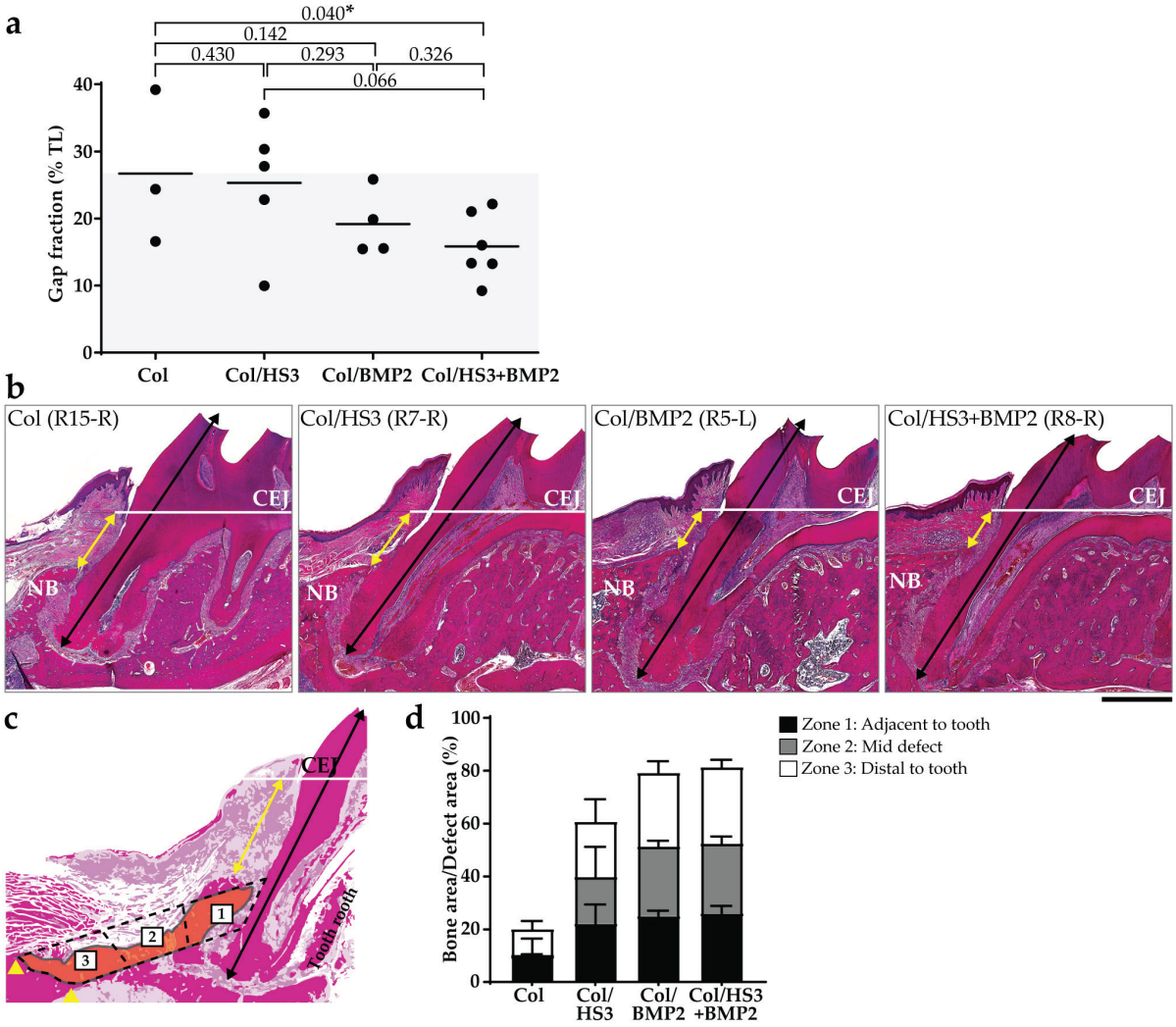

Fig. 4. Alveolar crest gap fraction. (**a**) Graph illustrating gap fraction (% of tooth length). The shading represents two ranges of gap fraction value defined as lower or higher than the mean value of the collagen alone group (0-26.7 and 26.8-100). (**b**) Representative H&E-stained histology images from each treatment group. White line indicates the CEJ; yellow line indicates the gap between the regenerated alveolar crest to the CEJ; black line indicates the tooth length (cusp to apical tip); NB: new bone; scale bar = 1 mm. (**c**) Diagram illustrating the three zones and (**d**) graph showing the amount of new bone formed in each zone for every treatment group. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Table 4. Summary of the number of data points in each shading zone in Fig. 4a and descriptive statistics of the gap fraction data.

	Gap fraction (% TL)			
	Col	Col/HS3	Col/BMP2	Col/HS3 + BMP2
Shading zone				
26.8-100	33.3 % (1/3)	60 % (3/5)	0 % (0/4)	0 % (0/6)
0-26.7	66.7 % (2/3)	40 % (2/5)	100 % (4/4)	100 % (6/6)
Minimum	16.6	10.0	15.5	9.2
Maximum	39.2	35.7	25.9	22.2
Mean	26.7	25.3	19.2	15.8
Median	24.4	27.8	17.2	14.7
SD	11.5	9.8	4.9	5.0
Coefficient of variation	43.0	38.5	25.5	31.5

Table 5. Amount of new bone formed in each zone illustrated in Fig. 4d for every treatment group. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

	Bone area/defect area (%)		
	Zone 1	**Zone 2**	**Zone 3**
Col alone	10.0 ± 6.4	0.4 ± 0.2	9.7 ± 3.1
Col/HS3	22.2 ± 7.2	17.6 ± 11.4	21.0 ± 8.5
p (*vs.* Col alone)	0.026*	0.0135*	0.0555
Col/BMP2	25.0 ± 2.0	26.4 ± 2.2	27.9 ± 4.4
p (*vs.* Col alone)	0.0031**	<0.0001***	0.0778
Col/HS3 + BMP2	26.1 ± 2.8	26.4 ± 2.7	29.0 ± 2.7
p (*vs.* Col alone)	0.0005***	<0.0001***	0.0399*

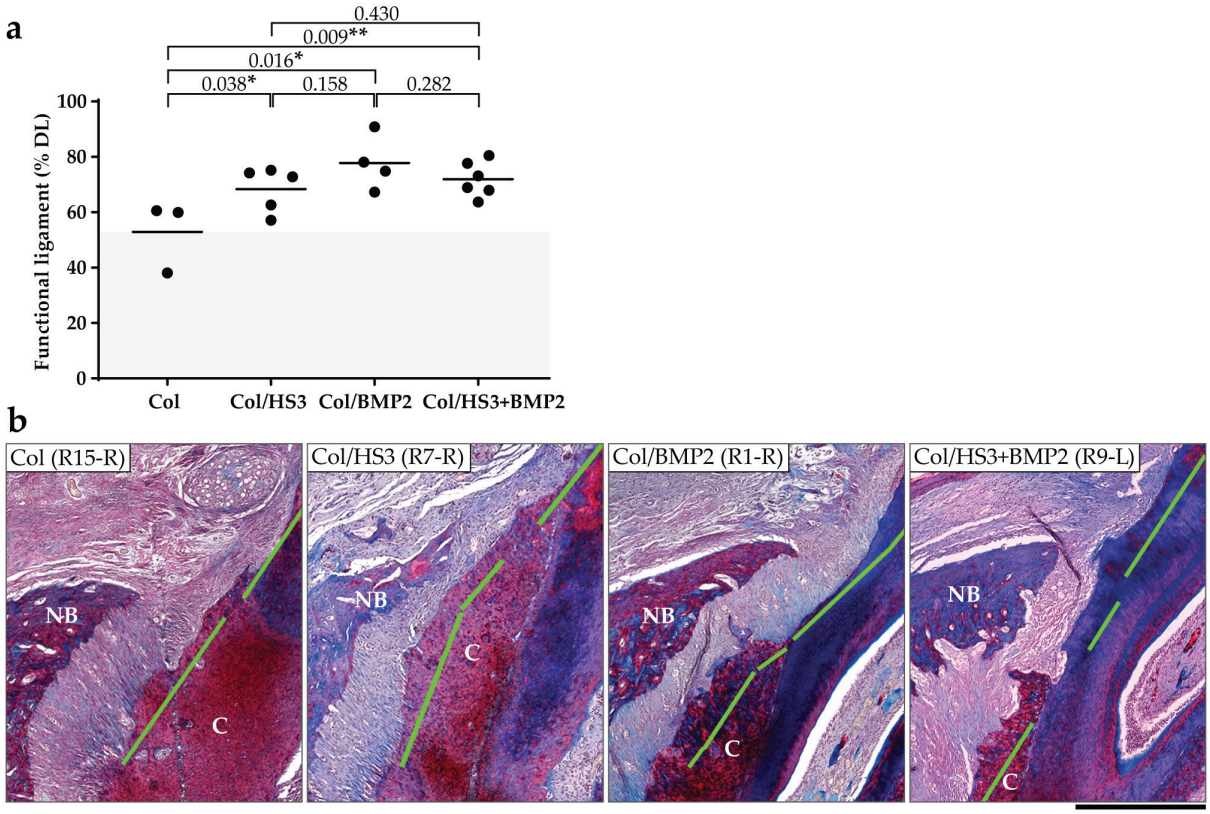

Fig. 5. Functional ligament. (**a**) Graphs illustrating the ratio of functional ligament to the defect length. The shading represents two ranges of values defined as lower or higher than the mean of the collagen alone group (0-52.9 and 53-100). *$p \leq 0.05$; **$p \leq 0.01$. (**b**) Representative Ralis-tetrachrome-stained histology images from each treatment group showing the length of newly regenerated functional ligament (marked by green lines). NB: new bone; C: cementum. Scale bar = 0.5 mm.

Table 6. Summary of the number of data points in each shading zone in Fig. 5a and descriptive statistics of the functional ligament data.

	Functional ligament (% defect length)			
	Col	**Col/HS3**	**Col/BMP2**	**Col/HS3 + BMP2**
Shading zone				
53-100	66.7 % (2/3)	100 % (5/5)	100 % (4/4)	100 % (6/6)
0-52.9	33.3 % (1/3)	0 % (0/5)	0 % (0/4)	0 % (0/6)
Minimum	38.1	57.1	67.3	63.7
Maximum	60.6	75.2	90.9	80.5
Mean	52.9	68.4	77.8	72.0
Median	59.9	72.8	76.5	71.0
SD	12.8	8.1	9.8	6.3
Coefficient of variation	24.2	11.8	12.7	8.8

Table 7. Summary of the number of data points in each shading zone in Fig. 6 and descriptive statistics of the epithelia apical growth data.

	Epithelial growth in apical direction (% defect length)			
	Col	Col/HS3	Col/BMP2	Col/HS3 + BMP2
Shading zone				
13.8-100	66.7 % (2/3)	20 % (1/5)	25 % (1/4)	16.7 % (1/6)
0-13.7	33.3 % (1/3)	80 % (4/5)	75 % (3/4)	83.3 % (5/6)
Minimum	8.1	1.1	0.6	2.3
Maximum	16.7	19.8	29.0	14.0
Mean	13.7	8.4	11.6	6.4
Median	16.3	6.5	8.5	6.1
SD	4.9	7.2	12.8	4.1
Coefficient of variation	35.4	85.2	110.5	64.7

molar and the original margins of the bone plate. For all treatments, the collagen sponge was no longer visible in the defect area, indicating a full degradation of the collagen implant. The extent of new bone formation can be clearly distinguished between the treatment groups, with the collagen alone treatment having the least bone regeneration, compared to the collagen/HS3, collagen/BMP2 and collagen/HS3 + BMP2 groups (Fig. 3**b**, left column). Where bone failed to regenerate inside the defect (collagen alone), a network of fibrous tissue was observed to fill the space. Of note, when either collagen/BMP2 or collagen/HS3 + BMP2 was used, bone marrow elements were more frequently observed within the defect that included the presence of adipose-like material. In comparison, treatment with collagen/HS3 resulted in the formation of more compact bone-like material that was less porous and continuous with the junction of the host bone. Unlike treatment with collagen alone, the addition of HS3, BMP2 or HS3 + BMP2 to collagen resulted in areas of new bone formation within the defect undergoing active bone remodelling characterised by numerous Harversian canals and cement lines (Fig. 3**b**, right column).

Alveolar crest gap fraction

In addition to alveolar bone regeneration, restoration of alveolar bone crest height immediately next to the tooth root was also examined. Alveolar bone crest height directly influences regeneration of the periodontium by providing an attachment point for periodontal ligament and preventing the down-growth of invading epithelium. The gap between the alveolar bone crest and the CEJ is referred to as the alveolar crest gap fraction and a smaller gap fraction represents improved restoration of the alveolar-bone crest. Treatment with collagen alone resulted in a mean gap fraction (% TL) of 26.7 ± 11.5 % (Fig. 4**a**; Table 4). Taking this value as a benchmark for crest recovery, treatment with either collagen/HS3 ($p = 0.430$) or collagen/BMP2 ($p = 0.142$) did not provide any improvement in crest restoration (Fig. 4**a**; Table 4). However, there was an improvement in the distribution of the data points following treatment with collagen/BMP2. The data show that gap fraction values in all defects (100 %) treated with collagen/BMP2 were below the mean response to collagen alone (Table 4). Notably, the combination of collagen/HS3 + BMP2 not only significantly reduced the gap fraction (15.8 ± 5 %, $p = 0.040$*) but also, similar to collagen/BMP2, all treatment outcome values were below the mean response to collagen alone.

Representative histological images illustrating the gap fraction for each treatment group are shown in Fig. 4**b**. These data show that treatment of alveolar bone defects with collagen alone can regenerate the alveolar crest in the region next to the tooth root to a level similar to collagen/HS3 or collagen/BMP2. However, restoration is mostly limited to the region adjacent to the tooth root. Importantly, treatment with collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 resulted in ~3 to 4-fold increase in new bone formation within the entire alveolar defect (Fig. 3). Therefore, to further evaluate the distribution of new bone within the alveolar defect, the defect was divided into three zones of equal area (Fig. 4**c**). The data show, of the new bone formed in the collagen alone group (19.6 ± 8.2 %, Table 2), only 0.4 ± 0.2 % was formed in zone 2 (the middle of the defect), while 10 ± 6.4 % was formed in zone 1 (adjacent to the tooth

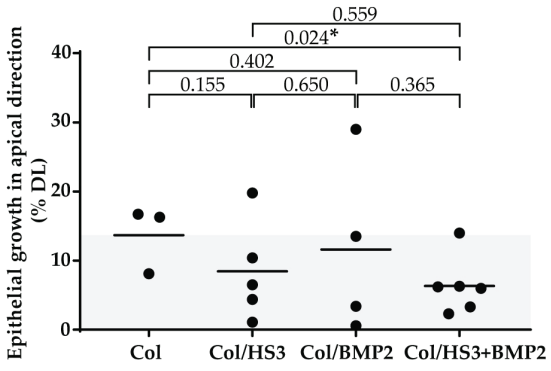

Fig. 6. Epithelial growth in apical direction. Graph illustrating the ratio of the epithelial growth in apical direction to the defect length. The shading represents two ranges of values defined as lower or higher than the mean of the collagen alone group (0-13.7 and 13.8-100).*$p \leq 0.05$.

root) and 9.7 ± 3.1 % was formed in zone 3 (furthest from the tooth root) (Fig. 4**d**; Table 5). In comparison, treatment with collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 resulted in equal distribution of new bone across the three zones of the defect (Fig. 4**d**; Table 5).

Regeneration of periodontal ligament and the extent of epithelial growth in the apical direction

The periodontal ligament is an essential part of the periodontium and functions to secure the tooth root into the alveolar socket. In the current model, the ligament, together with its associated alveolar-bone and tooth-root cementum, was removed when creating the defect. Therefore, any ligament observed post-surgery was newly regenerated. Moreover, only ligament bundles with an attachment angle > 60° to the cementum were considered functional (Fig. 2$\mathbf{b}_2$). When defects were treated with collagen alone, functional ligament regeneration was limited to 52.9 ± 12.8 % of the % DL) (Fig. 5; Table 2,6). The combination of collagen/HS3 improved functional ligament to 68.4 ± 8.1 % DL, a 1.3-fold increase compared to collagen alone (p = 0.038). Notably, all the functional ligament values (100 %) for collagen/HS3 were higher than the mean value for treatment with collagen alone (Fig. 5; Table 6). Treatment with collagen/BMP2 or collagen/HS3 + BMP2 further increased the length of function ligament to 77.8 ± 9.8 and 72 ± 6.3 % DL respectively, 1.5- and 1.4-times higher than collagen alone (p = 0.016* for BMP2 and p = 0.009** for HS3 + BMP2), but this was not statistically different from collagen/HS3 (Fig. 5**a**; Table 2,6). Paralleling the results for collagen/HS3, all functional ligament values (100 %) for collagen/BMP2 and collagen/HS3 + BMP2 were higher than the median value for collagen alone (Fig. 5**a**; Table 6). Of note, the periodontal ligament length from the uninjured side of the tooth (posterior side of the first molar) had an average length of 96 % DL (data not shown). Representative histological images illustrating the functional ligament for each treatment group is shown in Fig. 5**b**.

Completing the analysis of periodontal regeneration, the extent of epithelial tissue invading the periodontal milieu was assessed. It is generally agreed that epithelial downgrowth (apical) along the exposed tooth root prevents periodontal tissue regeneration. Treatment with collagen alone resulted in an epithelial growth of 13.7 ± 4.9 % DL (Fig. 6**a**; Table 2,7). When HS3 or BMP2 was added to collagen, the average epithelial growth reduced to 8.4 ± 7.2 and 11.6 ± 12.8 % DL, respectively, but this difference was not statistically significant when compared with the collagen alone (p = 0.155 for HS3 and 0.402 for BMP2) (Fig. 6**a**; Table 2,7). However, > 75 % of the values for collagen/HS3 or collagen/BMP2 were below the mean response to treatment with collagen alone (Fig. 6**a**; Table 7). Notably, treatment with collagen/HS3 + BMP2 significantly reduced epithelial growth to 6.3 ± 4.1 % DL (p = 0.024*) compared to collagen alone; however, this was not significantly different from collagen/HS3 or collagen/ BMP2 (Fig. 6**a**,**b**). Also, 83.3 % of the values for collagen/HS3 + BMP2 were lower than treatment with collagen alone. For benchmarking, epithelial growth along the uninjured side of the tooth averaged 3 % (data not shown).

Discussion

In this study, the intention was to investigate the effect of HS3 on periodontal regeneration in a rat maxillary intra-bony defect model. The data show that treatment of alveolar bone defects with a combination collagen/HS3 device significantly increased new alveolar bone formation by ~ three-fold compared to collagen alone. Notably, treatment with collagen/HS3 had equal efficacy for alveolar bone regeneration to collagen/BMP2. Treatment with collagen/HS3 + BMP2 was the only combination device to statistically increase alveolar crest height compared to collagen alone. Paralleling enhanced bone regeneration within the alveolar defect, treatment with collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 showed increased functional ligament restoration that was ~1.5-fold higher than treatment with collagen alone. Assessment of epithelial growth highlighted the inverse relationship between alveolar crest height and epithelial growth with collagen/HS3 + BMP2 performing significantly better than collagen alone.

Maintaining alveolar bone health is essential to providing long-term support for the dentition. In comparison to bone defects in the extremities that can heal spontaneously (Lim *et al.*, 2019), periodontal defects left untreated usually deteriorate (Cai *et al.*, 2018; Oortgiesen *et al.*, 2013; Yan *et al.*, 2015). There is accumulating evidence that bioactive mediators may prove useful for use in regeneration (Kaigler *et al.*, 2006; Werner and Grose, 2003). Several growth factors have shown promising outcomes in pre-clinical and clinical trials of periodontal regeneration (Lee *et al.*, 2010). Human recombinant BMP2, a bioactive mediator delivered with various bone graft substitutes or collagen sponges, has been studied for its efficacy on alveolar bone healing in orthopaedic, craniofacial and oral settings (Boyne *et al.*, 2005; Butura and Galindo, 2014; Jovanovic *et al.*, 2007; Sigurdsson *et al.*, 1995; Wikesjö *et al.*, 2001). Despite being able to regenerate alveolar bone, BMP2 use has been hindered by concerns over adverse events such as swelling, seroma, cystic bone formation and ectopic bone formation (Carragee *et al.*, 2011; Poynton and Lane, 2002). Growth factor use for tissue regeneration necessitates exogenous dosing to stimulate cell activity and tissue growth (Vasita and Katti, 2006). However, their short half-life *in vivo* and side-effect profile caused by the multiple- or high-doses needed for efficacy limits their therapeutic appeal (Chen *et al.*, 2009; Hughes *et al.*, 2006; Varkey *et al.*, 2004).

Strategies to reduce the dose of BMP2, or completely obviate its use all together, are therefore of critical importance. One common approach to reduce BMP2 dosage is to use carriers that contain crosslinked glycosaminoglycans such as heparin, HS or chondroitin sulphate (Andrews *et al.*, 2019; Hachim *et al.*, 2019; Hettiaratchi *et al.*, 2020; Yang *et al.*, 2012). However, it remains a challenge to obviate BMP2 while maintaining an improvement in bone regeneration. Heparan sulphate glycosaminoglycan sugar variants were developed that can bind to BMP2 (termed HS3) and prolong BMP2-mediated signals (Murali *et al.*, 2013). HS3 was tested in several animal models for bone regeneration and shown that bioscaffolds containing HS3 (concentration 100-1500 μg/cm^3 of defect) are capable of regenerating up to 55 % of the bone volume in a defect area (Bhakta *et al.*, 2018; Le *et al.*, 2019; Murali *et al.*, 2013; Rai *et al.*, 2015). In the current study, collagen/HS3 devices (625 μg/cm^3 of defect) were able to regenerate ~60 % of an alveolar bone defect within 6 weeks.

Scaffolds containing BMP2 have been previously evaluated for bone regeneration in various periodontal defect models (Selvig *et al.*, 2002; Sigurdsson *et al.*, 1996; Wikesjö *et al.*, 1999; Wikesjö *et al.*, 2003b; Wikesjö *et al.*, 2003c; Wikesjö *et al.*, 2004;). The data show that BMP2 dosing between 0.05 and 0.4 mg/mL was capable of inducing significant amounts of new bone formation in large animal models (Wikesjö *et al.*, 2003a). In the current study, 0.125 mg/cm^3 of BMP2 was added to a collagen scaffold and placed into alveolar defects in rats. The data corroborate the bone-healing efficacy of BMP2 in these earlier studies. Importantly the current study showed that treatment with collagen/HS3 (no BMP2) resulted in similar amounts of new bone formation to collagen/BMP2. Moreover, an increase in bone marrow-like elements containing adipose tissue was observed following treatment with collagen/BMP2 or collagen/HS3 + BMP2. These BMP2-related observations concur with the findings from an earlier canine alveolar defect model (Wikesjö *et al.*, 2003a). In contrast, it was observed that treatment with collagen/HS3 resulted in newly formed bone that appeared more compact and less porous. Also, the junction between the intact host bone and the newly regenerated bone was seen to be more continuous, and the defect margins less distinct. These data suggested that treatment with collagen/HS3 provided a regenerative milieu that was more physiologically tuned to support normal bone deposition and remodelling. It is possible that collagen/HS3 sequesters endogenous pro-healing factors such as BMP2 to generate BMP2-mediated osteostimulatory signals for neighbouring cells. Also, it is known that alveolar bone has a rich vascular supply, so providing a source of endogenous growth factors (such as BMP2) available for tissue regeneration. It is therefore possible that collagen/HS3 provides an enhanced scaffolding template that supports endogenous healing.

Ligament restoration and attachment was also examined as a functional assessment of periodontal repair. In all treatments, there was histological evidence of periodontal ligament regeneration and anchorage into the newly formed cementum-like tissue on the root surface. Notably, treatment with collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 significant improved functional ligament attachment compared with collagen alone. This contrasts previous data where treatment with BMP2 did not improve regeneration of a functionally-oriented periodontal ligament, despite showing improved bone regeneration (Lee *et al.*, 2010; Oortgiesen *et al.*, 2014). In the current study, improved functional ligament scores (up to 1.5 fold) paralleled improved bone regeneration scores (up to ~ 4 fold) following treatment with collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 compared to collagen alone. Moreover, there was clear evidence of a treatment-related effect on the distribution of new bone within the defect site (Fig. 4**d**,**e**). The data show bone regeneration following treatment with collagen/HS3, collagen/BMP2 or collagen/HS3 + BMP2 ~ equal across the defect space. In comparison, treatment with collagen alone demonstrates more occurrences of healing adjacent to the tooth root and at the opposing end of the defect, with little new bone in the central part of the defect. This highlights a lack of uniform bone filling following treatment with collagen alone that is addressed by the addition of HS3, BMP2 or HS3 + BMP2.

Further consideration and clinical testing of collagen/HS3 formulations for dental application is warranted. HS3 is an extracellular matrix heparan sulphate glycosaminoglycan material with increased affinity for BMP2 (Murali *et al.*, 2013) that is not adversely affected by gamma sterilisation (Smith *et al.*, 2018) and can be readily formulated with a wide range of materials (Bhakta *et al.*, 2018; Quang Le *et al.*, 2020; Rai *et al.*, 2015). Also, because collagen/HS3 is thought to bind, stabilise, and potentiate the effects of endogenously produced growth factors, support for periodontal regeneration is likely matched to reparative waves of growth factors. When complexing collagen/HS3 with exogenous BMP2 (collagen/HS3 + BMP2), the overall predictability of outcomes (coefficient of variation) was generally increased compared to collagen/BMP2, suggesting that HS3 acts to help mediate the BMP2 signals. However, controlled BMP2 dosing studies with collagen/HS3 devices are needed to establish the optimal level of complexation.

It is recognised that these findings are derived from a rodent alveolar-defect model, whereby the defect dimension and healing responses may deviate from human clinical cases. Nevertheless, the data and observations of the current study provide the opportunity to direct future clinical studies of periodontitis. Here, collagen/HS3 is established as a promising treatment for periodontitis-related alveolar bone loss with enhanced functional recovery.

Conclusions

Treatment of periodontal defects with trauma to the alveolar bone, root cementum and periodontal ligament with a collagen/HS3 combination device enhanced periodontal regeneration to extents comparable with collagen/BMP2. These data highlight the potential application of collagen/HS3 as a device for the clinical treatment of periodontitis-related alveolar tissue loss.

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Discussion with Reviewers

Reviewer 1: Do you have an idea about the concentrations of BMP2 (and presumably other growth factors) that is sequestered in the HS3 matrix? Are the concentrations so low that – although causing the desired effects – unwanted side effects are less than when using BMP2?

Authors: Future studies will seek to determine the *in vivo* binding capacity of collagen/HS3 matrices for endogenous BMP2. This will also help inform the design of future safety/tolerability studies.

Reviewer 2: Do the authors think it is possible to find out what other endogenously produced factors (besides BMP2) are retained in the HS3 sugar to help induce the tissue regeneration?

Authors: We have previously assayed other growth factors, including FGF2, VEGF and PDGFbb, for their ability to bind HS3 (Murali *et al*., 2013). We found that although HS3 could bind to these growth factors, the binding affinity is much weaker compared to its binding to BMP2. This is because HS3 was isolated based on its affinity for BMP2 (peptide affinity chromatography). In order to sequester other growth factors, we have designed other chromatography processes to pull out the more selectively bound heparan sulphate species, such as heparan sulphate bound to FGF2 (Ling *et al*., 2020 – additional reference), VEGF165 (Poon *et al*., 2018 – additional reference) and vitronectin (Yap *et al*., 2018 – additional reference).

Reviewer 3: One of the major issues with the clinical use of BMP-2 have been its pleiotropic side effects. What side effects may be associated with BMP-2 binding heparan sulphate and how do they compare to those reported when using BMP-2?

Authors: We have tested HS3 in several animal models such as rabbit ulna defect (Murali *et al*., 2013; Rai *et al*., 2015), rat intramuscular (Bhakta *et al*., 2018), rat cranial defect (Le *et al*., 2019). So far, we have not observed any noticeable side effect related to the use of HS3.

Additional References

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