Comparison of acidic fibroblast growth factor on collagen carrier with calcium hydroxide as pulp capping agents in monkeys

Li Z, Sae-Lim V. Comparison of acidic fibroblast growth factor on collagen carrier with calcium hydroxide as pulp capping agents in monkeys.

Abstract – Acidic fibroblast growth factor (aFGF) has been shown to facilitate wound healing by stimulating fibroblast proliferation and angiogenesis. It has also been reported to possess a powerful anti-apoptotic function This study compared the histological pulp responses to aFGF on collagen carrier and $Ca(OH)_2$ placed on the mechanically exposed dental pulp in monkeys at two observation periods. Thirty-six teeth with pulp exposures were distributed into three groups according to the capping agents used prior to application of the coronal seal: collagen-based matrix carrier (group 1), aFGF on the collagen-based matrix carrier (group 2) and aqueous calcium hydroxide [$Ca(OH)_2$] paste (group 3). Specimens were harvested at 6 and 13 weeks postoperatively and prepared for hematoxylin and eosin, and Gram staining. Histological qualitative evaluation of pulp responses were performed under the light microscope following criteria modified from Cox et al. (17) and Hu et al. (18). Semi-quantitative analysis was also carried out using Kruskal–Wallis and Mann–Whitney *U*-tests. There was neither negligible inflammatory infiltrates with no bacteria present in the three groups at both timings, nor was there any significant difference in the soft tissue organization among the three groups at or between the 6- and 13-week observation periods. At 6 weeks, the hard tissue barrier produced by $Ca(OH)_2$ group (1.040 ± 0.089) was significantly more superior than aFGF/collagen carrier group (1.930 ± 0.825) ($P = 0.030$) as well as collagen carrier group (3.142 ± 1.069, $P = 0.018$). At 13 weeks, both aFGF/collagen carrier group (1.214 ± 0.485) and the collagen carrier group (1.457 ± 0.814) produced significantly better hard tissue barrier ($P = 0.040$ and $P = 0.017$, respectively) than earlier timing. However, these two groups did not induce significantly improved hard tissue barrier compared to that produced by aqueous $Ca(OH)_2$ paste which stimulated matrix secretion in a polar tubular dentin-like pattern.

Zhimei Li, Varawan Sae-Lim

Department of Restorative Dentistry, Faculty of Dentistry, National University of Singapore, Singapore

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Varawan Sae-Lim, Department of Restorative Dentistry, Faculty of Dentistry, National University of Singapore, Lower Kent Ridge Rd., Singapore 119260, Republic of Singapore
E-mail: rsdvsl@nus.edu.sg

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Odontoblasts, in close proximity to peripheral capillaries, are responsible for dentinogenesis (1). Upon loss or injury, the pulpal cells (2, 3) undergo proliferation and differentiation, giving rise to new odontoblast-like cells at the exposed pulp periphery in an attempt to re-establish pulp homeostasis (4), resulting in reparative dentinogenesis.

Calcium hydroxide [$Ca(OH)_2$] has been widely used as a capping agent on exposed dental pulp because of its ability to induce dentin bridge formation (5). There have been concerns over damage to the underlying pulp tissues probably related to its high pH (6). The tunnel defects demonstrated within such $Ca(OH)_2$-induced dentin bridge (7) also suggest that an incomplete barrier is formed.

Application of signaling molecule-based delivery systems has been proposed to be a potential regenerative pulp treatment strategy in reconstituting normal pulp tissue architecture and achieving reparative dentinogenesis (8). Collagen matrix is reported to be an effective repair accelerator in early-stage wound healing (9). Acidic fibroblast growth factor (aFGF) is recognized as a mitogen and an angiogenic factor (10). Early angiogenesis improves wound healing by facilitating the exchange of essential elements between interstitial fluid and blood plasma (11). The presence of numerous blood vessels, with the resultant increased oxygen tension, significantly enhances cellular differentiation (12). In addition, aFGF has recently been reported to have anti-apoptotic function (13) which speculatively allows for conducive pulpal healing. The objective of this study was to compare histologically the effects of aFGF on collagen carrier and $Ca(OH)_2$ paste on pulp healing responses in a nonhuman primate model.

Materials and methods

Animal and sample preparation

The animal surgical experiments were performed in accordance with the International Guiding Principles for Animal Research (14) after the approval by the Review Committee of the Animal Holding Unit, National University of Singapore was sought.

Non-carious and periodontally sound mature incisors and mandibular posterior teeth from nine male *Macaca fascicularis* aged 4–6 years, weighing 3.45–5.10 kg were selected. All the experimental procedures were performed under general anesthesia. Pre-anesthetic sedation with ketamine (5 mg kg^{-1} body weight) and atropine sulfate (0.05 mg kg^{-1} bodyweight) was administered intramuscularly. Anesthesia was induced by intravenous Thiopental (15 mg kg^{-1} body weight) and maintained by Isoflurane (1.00–1.25%) via oral intubations. Intra-operatively, the monkeys were given intravenous normal saline and postoperatively, standard monkey chow (Astra-Ewos, Sodertalje, Sweden) and amoxicillin (Betamox LA, Norbrook Lab Ltd, Newry, UK) at a dosage of 15 mg kg^{-1} body weight and 0.01 mg kg^{-1} Buprenorphine hydrochloride (Temgesic Injection, Schering Plough, Hull, UK) once a day.

Experimental procedures

'Class I' cavities with approximately 1.0 × 1.0 mm^2 pulp exposures were prepared with a sterile high speed carbide round bur no. 1. The exposed pulp was then copiously irrigated with sterile water prior to achieving hemostasis. The following materials were placed directly over the exposed pulp of the designated teeth according to the standard protocol for pulp capping procedure (15) prior to the coronal seal with Intermediate Restorative Material (IRM) (Dentsply Caulk, Milford, DE, USA) in the following groups:

1. Collagen-based matrix carrier. Sterile bioabsorbable type-I collagen membrane (Biomend, Calcitek, Carlsbad, CA, USA) of dimension 1.0 × 1.0 mm^2 moistened with 5 μl of sterile water;
2. aFGF on collagen matrix carrier. Approximately 5 μl of aFGF (Biosource, California, CA, USA) at the concentration of 0.08 mg ml^{-1}, equivalent to 0.4 μg of aFGF was carried on the 1.0 × 1.0 mm^2 collagen membrane; and
3. Aqueous $Ca(OH)_2$ paste. Estimated 0.4 μg of $Ca(OH)_2$ (Calasept; Nordiska Dental, Angelholm, Sweden) powder mixed into paste form with 5 μl sterile water.

Specimen processing

At 6 and 13 weeks, the animals were killed. Transcardiac perfusion with 4% phosphate buffered paraformaldehyde (pH 7.4) via left ventricle was performed under deep anesthesia of an overdose of intravenous sodium pentobarbital at 100 mg kg^{-1} bodyweight. Mandibular and the maxillary jaw blocks containing the experimental teeth were removed. Immediately after the harvest, the apical third of the roots were resected to allow optimal postfixation in the 10% formaldehyde. They were subsequently decalcified with 10% formic acid and paraffin-embedded for histological evaluation. Samples were serially longitudinally sectioned at 5 μm thickness on a rotary Microtome (Reichert Histo-Stat, Leica, Germany). At each 50 μm interval, two sections were mounted on glass slides. One section was stained with hematoxylin and eosin, while one other section was left unstained for supplementary examination, if deemed necessary. In addition, Gram staining was performed at every 250 μm interval for the presence of bacteria. In total, approximately 15–20 sections were selected from each tooth for evaluation (16).

Histological evaluation

Under the light microscope (GalerTM III, Cambridge Instruments, Germany), pulp responses were evaluated blindly at magnifications of ×20, ×40, ×100 and ×200 based on criteria modified from that described by Cox et al. (17) and Hu et al. (18).

Inflammatory response scores

1 No scattered inflammatory cells present at or beneath pulp exposure site;
2 Acute or chronic inflammatory lesions, characterized by polymorphonuclear or mononuclear leukocytes at the exposure site;
3 Severe inflammatory lesions manifested as abscesses or dense infiltrate of polymorphonuclear leukocytes involving one-third or more of the coronal pulp;
4 Total pulp necrosis.

Soft tissue organization scores

1 Normal or almost normal tissue morphology at the exposure site and throughout the pulp;
2 Lack of complete tissue morphology at the exposure site, but the remaining pulp tissue was normal;
3 Necrosis in at least the coronal third or more of the tooth pulp.

Bacterial staining scores

1 No evidence of bacteria in the prepared cavity, exposed dentinal tubules or the pulp;
2 Bacterial staining along the cavity walls;
3 Bacterial staining along the cavity walls, within the exposed dentinal tubules and/or pulp.

Reparative tissue scores over pulp exposure site

1 Complete barrier with tubular dentin-like structure at the pulpal front;
2 Complete barrier with amorphous osteo-dentin-like structures;
3 Incomplete hard tissue repair; and
4 No repair.

Statistical analysis

The occurrence of the different criteria under each category of tissue reactions was expressed as a mean score for each section. The average score of each category of tissue reactions in each tooth was then calculated from the mean of the total number of sections. The mean and standard deviation of each category of tissue reactions were then calculated for each treatment group. The latter were then analyzed using three-way Kruskal–Wallis and Mann–Whitney *U*-tests. Significance level was set at $P < 0.05$.

Results

The monkeys tolerated the operative procedures well. Teeth with fractured crowns or compromised coronal seal at the time of kill were excluded from evaluation. This lead to a final sample of 36 teeth consisting of 14 teeth in group 1 (collagen carrier), 15 teeth in group 2 (aFGF/collagen carrier) and seven teeth in group 3 [$Ca(OH)_2$ paste].

The results for each treatment group are summarized in Tables 1 and 2. There was a negligible amount of inflammatory response with few or no inflammatory cells observed at the pulp exposure site in the majority of the sections which were not significantly different among the three groups at 6 weeks ($P = 0.424$; Fig. 1) and 13 weeks ($P = 0.852$; Fig. 2) as well as between the two observation time intervals within each of the three groups ($P = 0.710$, $P = 0.955$ and $P = 1.000$, respectively).

Similarly for the soft tissue organization, there was no significant difference among the three groups at both 6 and 13 weeks postoperatively ($P = 0.145$ and $P = 0.230$, respectively) as well as between the 6 and 13 weeks within each same groups 1, 2 and 3 ($P = 0.613$, $P = 1.000$ and $P = 0.053$, respectively). No bacterial penetration along the cavity wall could be detected in any specimen.

At 6 weeks, the $Ca(OH)_2$ group (group 3) resulted in complete hard tissue barriers with some displaying irregular osteodentin-like structures (1.040 ± 0.089; Fig. 1). Comparatively, the reparative tissue produced in the group 1, collagen carrier group (3.142 ± 1.069) and the group 2, aFGF/collagen carrier group (1.930 ± 0.825) were significantly more inferior ($P = 0.018$ and $P = 0.030$, respectively). There was mostly incomplete repair with amorphous tissue in the collagen carrier group (Fig. 3) while in the aFGF/collagen carrier group, the majority of the osteodentin formed were interspersed with cellular inclusions (Fig. 4).

At a longer observation period of 13 weeks, the aFGF/collagen carrier group (1.214 ± 0.485) and collagen carrier group (1.457 ± 0.814) had significantly better reparative tissue repair when compared with the 6-week period ($P = 0.040$ and 0.017, respectively). Although all three groups appeared to produce hard tissue with some form of tubular dentin-like structures at the pulpal front ($P = 0.695$), $Ca(OH)_2$ uniquely stimulated tubular matrix secretion in a polar dentin-like pattern (Fig. 5). On the other hand, aFGF/collagen carrier group produced some tubular matrix structures liken dentin at some peripheral pulpal front aligned with cuboidal to columnar odontoblast-like cells whereas, the tubular-like structures lined with flattened cells were irregular in pattern (Fig. 2). In the collagen matrix

Table 1. Statistical comparisons of mean scores for different categories of tissue reactions among three experimental groups at 6 and 13 weeks, respectively

Weeks	Histological evaluations	Groups	*n*	Mean ± SD	Kruskal–Wallis test	Group comparison	Mann–Whitney *U*-test
6	Inflammatory response	1	7	1.000 ± 0.000	0.424	1 vs 2	0.710
		2	7	1.142 ± 0.377		1 vs 3	1.000
		3	5	1.000 ± 0.000		2 vs 3	0.755
	Hard tissue barrier	1	7	3.142 ± 1.069	0.009*	1 vs 2	0.053
		2	7	1.930 ± 0.825		1 vs 3	0.018*
		3	5	1.040 ± 0.089		2 vs 3	0.030*
	Soft tissue	1	7	1.065 ± 0.173	0.145	1 vs 2	0.318
		2	7	1.428 ± 0.534		1 vs 3	0.755
		3	5	1.000 ± 0.000		2 vs 3	0.268
13	Inflammatory response	1	7	1.143 ± 0.377	0.852	1 vs 2	0.955
		2	8	1.041 ± 0.116		1 vs 3	0.889
		3	2	1.000 ± 0.000		2 vs 3	0.889
	Hard tissue barrier	1	7	1.457 ± 0.814	0.695	1 vs 2	0.867
		2	8	1.214 ± 0.485		1 vs 3	0.667
		3	2	1.000 ± 0.000		2 vs 3	0.711
	Soft tissue	1	7	1.543 ± 0.461	0.230	1 vs 2	0.955
		2	8	1.498 ± 0.458		1 vs 3	0.222
		3	2	1.000 ± 0.000		2 vs 3	0.178

Group 1, collagen carrier; group 2, aFGF/collagen carrier; 3, $Ca(OH)_2$.
*$P < 0.05$.

Table 2. Statistical comparisons of mean scores of different categories of tissue reactions between 6- and 13-week time intervals

Histological evaluation	Group	6 weeks *n*	6 weeks Mean ± SD	13 weeks *n*	13 weeks Mean ± SD	Mann–Whitney *U*-test
Inflammatory response	1	7	1.000 ± 0.000	7	1.143 ± 0.377	0.710
	2	7	1.142 ± 0.377	8	1.041 ± 0.116	0.955
	3	5	1.000 ± 0.000	2	1.000 ± 0.000	1.000
Hard tissue barrier	1	7	3.142 ± 1.069	7	1.457 ± 0.814	0.017*
	2	7	1.930 ± 0.825	8	1.214 ± 0.485	0.040*
	3	5	1.040 ± 0.089	2	1.000 ± 0.000	0.857
Soft tissue	1	7	1.065 ± 0.173	7	1.543 ± 0.461	0.053
	2	7	1.428 ± 0.534	8	1.498 ± 0.458	0.613
	3	5	1.000 ± 0.000	2	1.000 ± 0.000	1.000

Group 1, collagen carrier; 2, aFGF/collagen carrier; 3, $Ca(OH)_2$.
*$P < 0.05$.

group, the tubular structures were sparse and the reparative tissue was more heterogeneous (Fig. 6). In addition, there was also evidence of the apposition of mineralized tissue along the lateral wall of the pulp cavity adjacent to the exposure site in the aFGF/collagen carrier group as in the collagen carrier group (Figs 2–4 and 6).

Discussion

The dentin-pulp complex is recognized as possessing inherent healing capacity in response to damage caused by either physical or chemical injuries (2). In our study, odontoblasts were destroyed during the surgical procedure of pulp exposure. Following the inadvertent initial inflammatory responses, negligible or no inflammation was observed in all the three groups both at 6 and at 13 weeks postoperatively (Table 2). Acute inflammation which occurred in response to mechanical pulp exposure resolved with time in all the three groups in the absence of bacteria shown to be the primary cause of persistence inflammation (19), as confirmed under the gram stain. This further substantiated the aseptic technique employed throughout our operative procedures and the good seal provided by the IRM (20). The reorganization of the pulp connective tissue and the formation of the hard tissue assumably was formed by the residing cells within the dental pulp (21).

The use of calcium hydroxide as a pulp capping agent has been extensively studied. Despite the only two samples left in the latter timing of 13 weeks, our uniform histologic outcome of the aqueous $Ca(OH)_2$ group, indicated by the nil standard deviation in most of the evaluation categories (Table 1), collaborated well with the earlier studies (22). There was typical exhibition of an alkalinity-induced necrotic zone with hard tissue barrier formed at some distance from the exposure site (Fig. 1). The previously formed osteodentin-like hard tissue at 6 weeks (Fig. 1) progressed with time to demonstrate complete regular tubular dentinal-like matrix barrier pulpally lined with odontoblast-like cells at 13 weeks (Fig. 5). Such mechanism for reparative dentinogenesis is not fully elucidated. The elevated extracellular calcium ion at the trauma site has been speculated to contribute to the dynamic interactions between the modified microenvironment (23) and odontoblast-like cells, allowing alignment against the calcium proteinate matrix during which dentin-

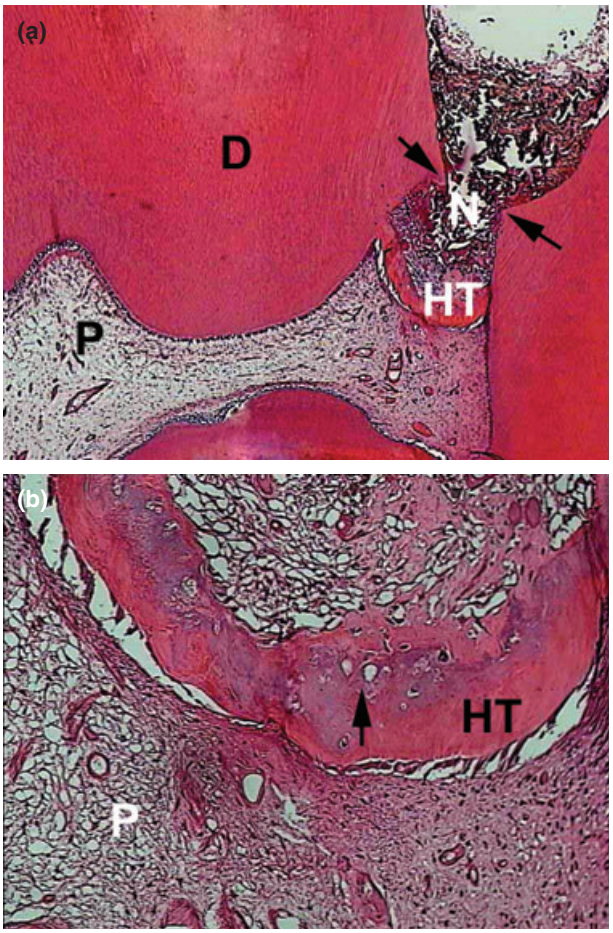

Fig. 1. Representative histologic photomicrographs of the $Ca(OH)_2$ group at 6-week observation period. Overview [(a) original magnification ×20, H&E staining; D, dentin; P, pulp; N, necrotic layer; HT, hard tissue] illustrating the pulpally convex hard tissue barrier formed at a distance away from the exposure site (dark arrows) with no evidence of inflammatory cells within the remaining underlying pulp tissue. Higher magnification [(b) ×100] demonstrating cellular inclusions with porosity (open arrow) was mainly localized in the upper part of the amorphous osteodentin-like hard tissue barrier formed.

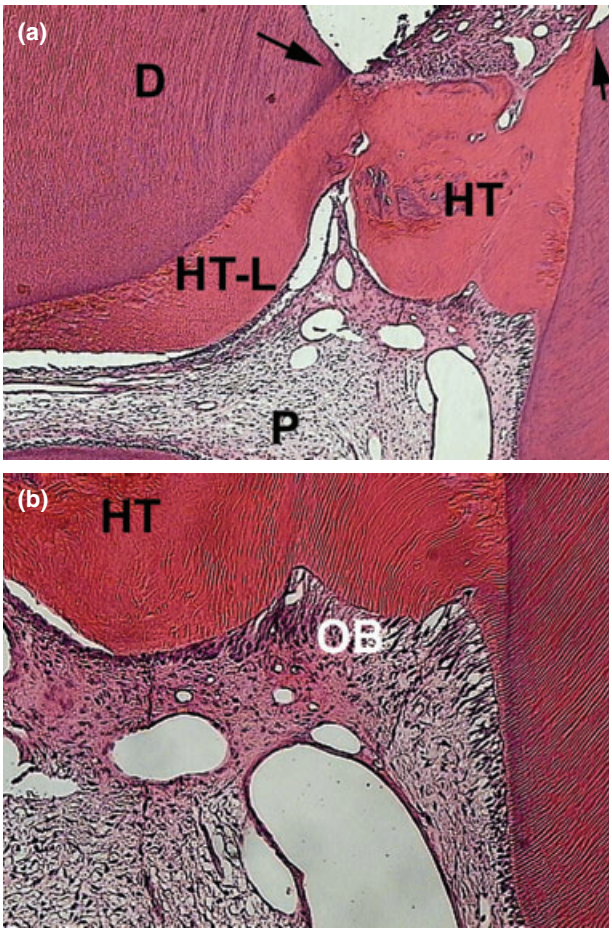

Fig. 2. Representative histologic photomicrographs of the aFGF/collagen carrier group at 13-week observation period. Overview [(a) original magnification ×40, H&E staining; D, dentin; P, pulp; HT, hard tissue] illustrating heterogeneously mineralized hard tissue barrier formed at a level closer to the exposure site (dark arrows) above the underlying reorganized pulp tissue free of inflammatory cells. There is observed apposition of mineralized tissue along the lateral wall of the pulp cavity (HT-L) below the cut dentin. Higher magnification [(b) ×100] demonstrating part of the pulpal front of the hard tissue barrier with tubular dentin-like appearances and the columnar or cuboidal odontoblast-like cells (OB) aligning beneath, while other parts showing atubular or irregular dentin-like structures lined with cuboidal or flattened cells.

ogenesis take place (5) with the reported increased expression of mineralized matrix proteins such as osteopontin and bone morphogenic protein-2 (24).

On the other hand, despite the clean mechanical pulp exposure model, the bioactive aFGF investigated did not appear to produce hard tissue repair of similar quality to that capped with aqueous calcium hydroxide paste. This seemed to substantiate the previous *in vitro* observation that aFGF alone could merely promote cell polarization but not odontoblastic differentiation (25) although it enhances fibroblast proliferation (26) and angiogenesis in the early phase of wound healing (27). It has been suggested that the previously formed irregular extracellular osteo-dentin-like hard tissue matrix at 6 week might have served as a reservoir for cytokines such as Transforming Growth Factor-β (TGF-β) (28). The acidity of the aFGF has also been shown to play a role in the mineralization of predentinal matrix (29). Speculatively, it is plausible that the regular tubular dentin-like hard tissue observed at 13 weeks might be attributed in part by the synergistic interaction between the intrinsically released TGF-β and the extrinsically applied aFGF leading to induction of odontoblast differentiation and extracellular matrix mineralization as has been shown in the dental papillae culture (25, 30). Although possible, it is uncertain, however if more regular tubular dentin

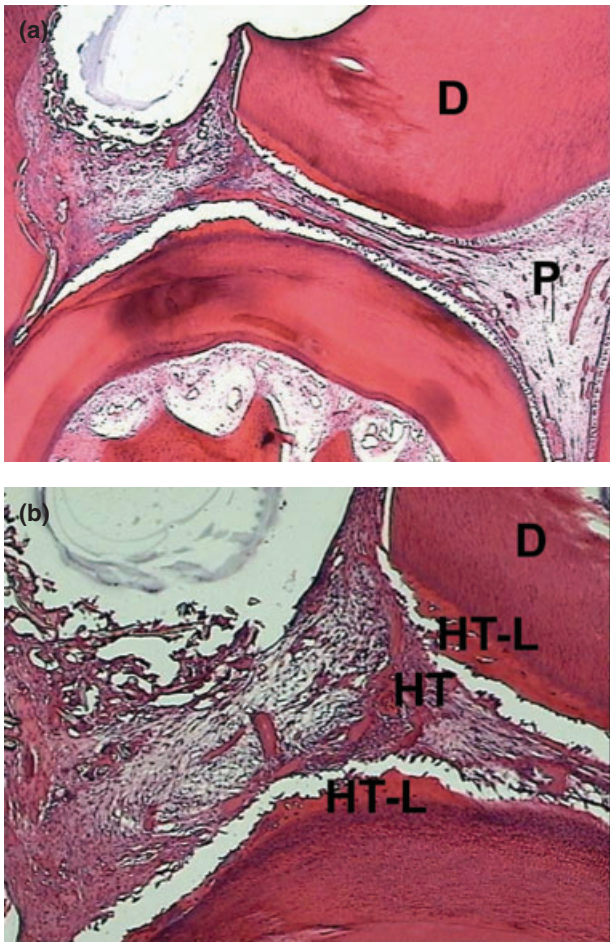

Fig. 3. Representative histologic photomicrographs of the collagen carrier group at 6-week observation period. Overview [(a) original magnification ×20, H&E staining; D, dentin; P, pulp; HT, hard tissue] and higher magnifications [(b) ×40] illustrating incomplete reparative tissue repair and the inadequately organized underlying pulp tissue. There is also small amount of mineralized tissue apposing along the lateral wall of the pulp cavity (HT-L) below the cut dentin.

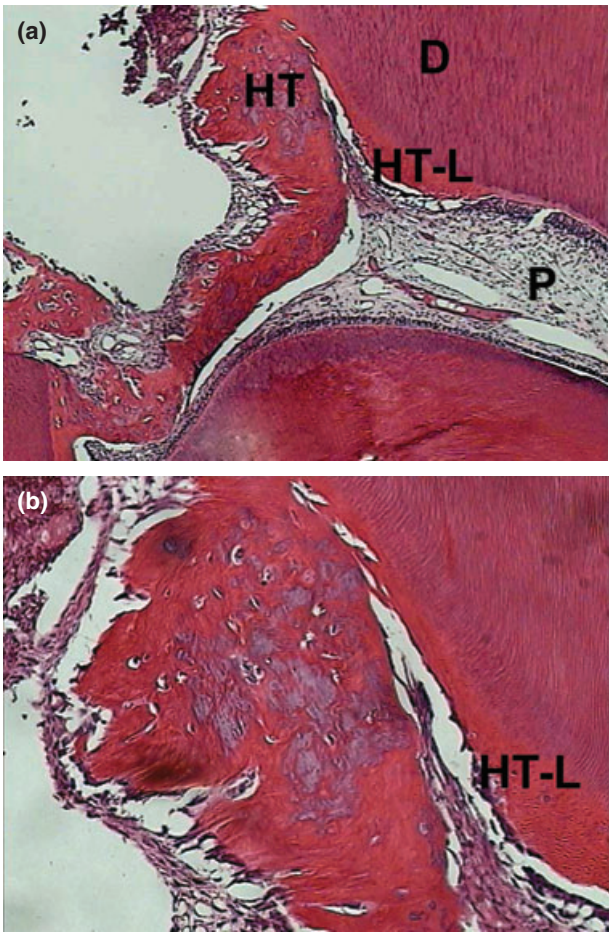

Fig. 4. Representative histologic photomicrographs of the aFGF/collagen carrier group at 6-week observation period. Overview [(a) original magnification ×40, H&E staining; D, dentin; P, pulp; HT, hard tissue] illustrating amorphous hard tissue with cellular inclusions formed at the exposure site. There is observed apposition of mineralized tissue along the lateral wall of the pulp cavity (HT-L) below the cut dentin. Higher magnification [(b) ×100] demonstrating cellular inclusions (open arrow) throughout the osteodentin-like hard tissue barrier formed.

would be formed with a longer observation period than 13 weeks. Apoptosis of dental pulp cells is reportedly induced during wound healing and regeneration (31, 32). In this study, the anti-apoptotic function of aFGF did not appear to favorably influence the pulpal healing and dentinogenesis.

The scarcely mineralized fibrous tissue repair formed at 6 weeks beneath the Biomend collagen membrane carrier (Fig. 3) appeared to confirm that type I collagen alone has limited modulating capacity on odontoblastic differentiation (33) despite the contention that the extrinsically applied collagen might serve as an initial scaffold for early competent pulpal cell attachment, enhancing further collagen matrix formation (34). The subsequent presentation of a heterogeneous hard tissue barrier lined with flattened cells at 13-week (Fig. 6) might also be a result of irregular proliferation of underlying pulp cells possibly related to large blood clots beneath the collagen membrane (35).

The absence of mineralized tissue apposition on the lateral wall of the pulp cavity when $Ca(OH)_2$ was used as a pulp capping agent (Fig. 5) appeared to suggest its functional uniqueness. The high alkalinity of $Ca(OH)_2$ induces self-limiting necrosis of the subjacent pulp tissue leaving the surviving pulp sufficiently intact to bring about reorganization and maintenance of normal pulp integrity (35, 36). In contrast, the presence of mineralized tissue formed along the lateral wall of the pulp cavity in the aFGF/collagen carrier and the collagen carrier

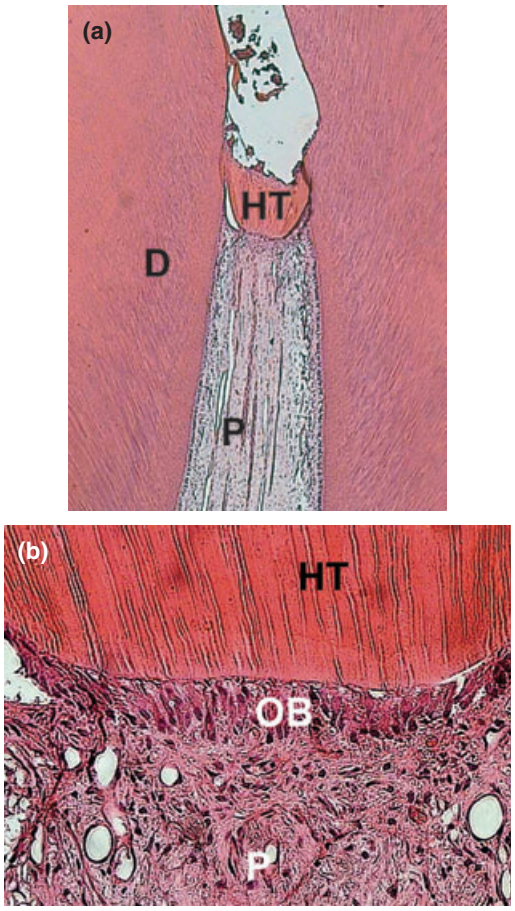

Fig. 5. Representative histologic photomicrographs of the $Ca(OH)_2$ group at 13-week observation period. Overview [(a) original magnification 20, H&E staining; D, dentin; P, pulp; HT, hard tissue] illustrating hard tissue barrier formed with no evidence of inflammatory cells within the remaining underlying pulp tissue which appeared to be normally reorganized. There is no apposition of mineralized tissue along the lateral wall of the pulp cavity (HT-L) below the cut dentin. Higher magnification [(b) ×200] demonstrating the pulpal front of the hard tissue barrier with tubular dentin-like structures and odontoblast-like cells (OB) aligning beneath.

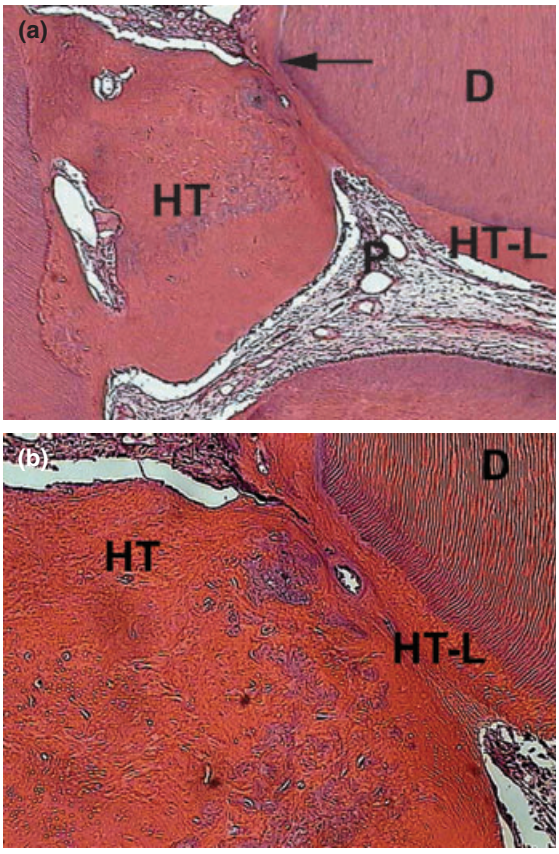

Fig. 6. Representative histologic photomicrographs of the collagen carrier group at 13-week observation period. Overview [(a) original magnification ×40, H&E staining; D, dentin; P, pulp; HT, hard tissue] illustrating heterogeneously mineralized hard tissue barrier formed at the exposure site (dark arrows) lined with cuboidal or flattened cells above the reorganizing underlying pulp tissue free of inflammatory cells. There is observed apposition of mineralized tissue along the lateral wall of the pulp cavity (HT-L) below the cut dentin. Higher magnification [(b) ×100] demonstrating part of the irregular dentin-like hard tissue barrier with cellular inclusions.

groups might indicate the compromised pulp status in the region. This calcification might consequently coalesce with the hard tissue barrier leaving a junction that potentially might not be imperviously sealed as previously reported with other pulp capping agents (35).

The arbitrary amount of aFGF used was an apparent limitation in this study. Nevertheless, inference was made to the minimal concentration of aFGF that would result in the peak growth rate in cell culture (37) or elicit detectable mitogenic effect on DNA synthesis, ALP activity and induction of odontoblast polarization (38, 39). The extent of the functional aFGF supposedly largely depends upon the unclear binding and release kinetics of aFGF on collagen carrier which is of further interest. The current aFGF concentration of 0.08 mg ml^{-1} however, appeared to have exerted an observable morphogenetic influence on the underlying pulpal cells manifesting a distinct dentinogenic pattern and the rate of hard tissue barrier repair different from the calcium hydroxide group (40). Nevertheless, the dynamic mechanisms of dentin bridge formation, as part of the consequence of sophisticated interplays of reciprocal factors (23) in an attempt to reestablish pulp homeostasis after injury (4), still remains largely an unknown domain. The postulation that capping agents (31) and other extrinsic insults (32) appeared to modulate the induction of apoptosis in pulp cells via pathway such as that of c-Jun N-terminal kinase (JNK) might implicate that apoptotic cells be eliminated or inhibited by protective protein like heat-shock protein 70 (HSP70) (41) before homeo-

stasis and the initiation of reparative dentinogenesis occurs during pulp wound healing (42). It would be of much interest to ascertain how significant a role apoptosis plays in wound healing and regeneration of dental pulp.

Conclusions

Acidic fibroblast growth factor on collagen-based matrix carrier did not produce a significantly superior hard tissue barrier compared to pulp capping with aqueous calcium hydroxide paste.

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