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Histologic Response and Tenascin and Fibronectin Expression After Pulp Capping in Pig Primary Teeth With Mineral Trioxide Aggregate or Calcium Hydroxide

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Clinical Relevance

Mineral trioxide aggregate produces a lower initial inflammatory response than calcium hydroxide. However, both capping materials produce dentin barriers after seventy days.

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SUMMARY

This study evaluated the histological response and the expression of tenascin (TN) and fibronectin (FN) after pulp capping with mineral trioxide aggregate (MTA) or calcium hydroxide (CH). Class V cavities and pulp exposure were performed in 40 primary pig teeth. The

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pulps were capped with either MTA or CH, and the cavities were sealed with resin-modified glass ionomer cement. CH was used as a control. Seven and 70 days posttreatment, the animals were sacrificed and teeth were prepared for histological evaluation. TN and FN were detected by immunostaining. A severe inflammatory response was observed after 7 days in the CH group ($p<0.043$), while in the MTA group, a mild response was observed. Similar reparative dentin deposition was observed after 70 days for both groups ($p<0.005$). The expression of FN and TN was similar for both groups in the two periods evaluated. TN and FN were expressed during pulp reparative events, independently of the capping material.

INTRODUCTION

Restorative strategies have been developed using different capping materials to preserve pulp vitality and its function.[1] Calcium hydroxide (CH) has been employed for several decades with good clinical results,[2] and the histological response to direct capping with this material is the formation of a dentin barrier and soft-tissue reorganization over time.[3] Although CH has been considered the gold standard material for direct pulp capping,[4] some shortcomings have been described such as the lack of adhesion to dental structure, high solubility, and the presence of microleakage.[5]

To optimize the results of pulp capping, new materials have been employed. Mineral trioxide aggregate (MTA) used as a direct capping agent has elicited similar response in human sound pulps when compared with CH[6] and is considered a promising material for vital pulp therapy.[7] MTA is used in pediatric dentistry because of its biocompatibility and sealing ability.[8] Long-term clinical follow-up of direct pulp capping in deciduous teeth with MTA has demonstrated the material to be successful,[8] and calcified barriers were observed under MTA in primary teeth from pigs.[9] The complete mechanisms behind reparative dentinogenesis following direct pulp capping remain unclear, but the stimulation of pulp cells seems to be the key factor in pulp repair.[10] The dentin solubilization by CH[11] or MTA[12] could release growth factors, which might initiate differentiation of stem cells in odontoblast-like cells. Extracellular matrices of dentin and pulp may have a pivotal role in the dental pulp repair process.[13] The glycoproteins tenascin (TN) and fibronectin (FN) regulate the differentiation process leading to mineral deposition in the dental pulp.[14] These glycoproteins have a significant role during the differentiation process that might occur in human dental pulps capped with CH,[15] but the effect of MTA on their expression remains unknown. This study aimed to evaluate the morphological response and the immunohistochemical expression of TN and FN in pulps from primary pig teeth using MTA and CH as capping agents.

MATERIALS AND METHODS

Experimental Procedures

After approval by the local Ethics Committee of Animal Experimentation (March 2005), six male swine pigs (*Sus scrofa domesticus*) were selected that were 105 days (±10 days) of age and 40 kg (±5 kg) of weight. Central and lateral primary incisors and second and third lower primary premolars were used to obtain at least seven teeth per animal.

Clinical Procedures

Initially, animals underwent anesthesia with an intramuscular injection of an association of acepromazin, midazolam, and Cetamin, followed by sodium thiopental inhalation, and were maintained with intubation and administration of halothane in a universal vaporizer.

Class V cavity preparations were done using a diamond bur #1013 and a carbide bur #330 with high speed under refrigeration for enamel and dentin removal up to pulp exposure, with a size restricted to the bur diameter. Saline solution was used for hemostasia, and after bleeding control, the pulp was capped with MTA (Angelus, Londrina, PR, Brazil) or CH cement (Dycal, Dentsply, Petrópolis, Rio de Janeiro, Brazil). The cavities were then sealed with resin-modified glass ionomer (Vitremer, 3M Espe, St Paul, Minn, USA), and a light-curing unit XL2500 (3M) was used during the experiment. All of these clinical procedures took approximately two hours per animal, and the anesthetic postoperative recovery took another two hours. The procedures were carried out in the surgery room of a veterinary clinical hospital (Federal University of Pelotas), under the supervision of a veterinarian.

Forty teeth were used in this study; 26 teeth were capped with MTA (13 for each time interval), and as a control, 14 teeth were capped with CH (seven for each time interval). Three animals were sacrificed at

Table 1: *Scores Attributed to the Levels of the Histological Criteria Evaluated*[a]

Score	Criteria for the morphological analysis			
	Inflammatory response	**Soft-tissue organization**	**Reactionary dentin**	**Reparative dentin**
1	None: the pulp contained few inflammatory cells, or an absence of inflammatory cells associated with cut tubules of the cavity floor.	Normal: there was no injury, disruption, or loss of cell survival.	None: no evidence of additional dentin deposition at the injury site.	None: no dentin barrier formation.
2	Mild: the pulp had localized inflammatory cell lesions predominated by polymorphonuclear leukocytes or mononuclear lymphocytes.	Mild: there was a superficial loss of cell survival at the site of injury.	Mild: there was a mild increase in dentin deposition, constituted by a thin layer produced by the original odontoblasts.	Mild: there was some dentin deposition by the odontoblast-like cells at the exposure site, in focal areas.
3	Moderate: the pulp had polymorphonuclear leukocyte lesions involving more than one-third of the coronal pulp.	Extensive: there was an extensive loss of cell survival involving more the superficial cells.	Intense: there was deposition of a thick and uniform layer of reactionary dentin.	Intense: there was uniform dentin formation by the odontoblast-like cells at the exposure site.
4	Severe: the pulp tissue was largely necrotic, following chronic inflammatory cell injury.	—	—	—

[a] *ISO 7405 and adapted from Silva and others 2006.*[17]

seven days and the remaining three at 70 days. Teeth were extracted and fixed in 10% buffered formalin for 24 hours. Specimens were demineralized in 20% formic acid, serially sectioned (3 μm), and stained with hematoxylin and eosin. Histological sections were examined using criteria based on ISO 7405[16] and the work of Silva and others[17] (Table l). Two blinded calibrated examiners carried out the descriptive analysis, evaluating the following criteria: inflammation, tissue organization, reactionary dentin (adjacent to the exposure site, produced by primary odontoblasts),[17,18] and reparative dentin formation (under the exposure site, produced by new odontoblast-like cells).[17,19]

When disagreement occurred between the two blinded examiners, another evaluator was invited and a consensus was obtained.

Statistical Evaluation

Data were submitted to statistical analysis using the software SigmaStat for Windows 3.0 (SPSS Inc, Chicago, Ill, USA). The confidence level was set at 95% ($p<0.05$) and evaluation was completed using the nonparametric Kruskal-Wallis test.

Immunohistochemical Staining

Sections were deparaffinized in xylene, rehydrated in different concentrations of alcohol, and washed in phosphate-buffered saline (PBS). The sections were washed in methanol with 3% hydrogen peroxide (H_2O_2, 1:1; 2×5 minutes). Primary antibodies were diluted in PBS-containing 0.1% bovine serum albumin as 1:1200 dilutions for anti-FN (Dako, AIS, Glostrup, Denmark) or 1:3000 dilutions for anti-TN (Sigma Chemical Co, St Louis, Mo, USA). The sections were incubated, respectively, at 37°C for 120 minutes and at 4°C for 18 hours, followed by a 30-minute incubation with 1:100 dilutions for biotinylated goat anti-rabbit or anti-mouse secondary antibody (Dako). Specific immunostaining was visualized by incubation with 3,3′-diaminobenzidine tetrachloride containing 0.03% H_2O_2 for 3 minutes and washing with distilled water. The sections were counterstained with Mayer's hematoxylin solution, rinsed in running tap water, dehydrated in ethanol, cleared with xylene, and mounted. The negative control staining was done by omitting the exposure to the primary antibodies. The ubiquity of the expression of TN and FN in blood vessels was also used as positive internal control. A descriptive

Table 2: *Histological Evaluation of Pulp Response for the Two Capping Materials Used, Based on the Criteria and Scores Predetermined*

Variable	Score			
	1	2	3	4
Inflammatory response				
CH				
7 days	2	4	4	2
70 days	11	0	1	0
MTA				
7 days	9	1	2	0
70 days	12	0	0	0
Soft-tissue organization				
CH				
7 days	0	10	2	—
70 days	0	12	0	—
MTA				
7 days	0	12	0	—
70 days	0	12	0	—
Reactionary dentin				
CH				
7 days	2	8	2	—
70 days	0	12	0	—
MTA				
7 days	5	4	3	—
70 days	1	10	1	—
Reparative dentin				
CH				
7 days	9	3	0	—
70 days	0	0	12	—
MTA				
7 days	6	6	0	—
70 days	0	0	12	—

analysis and immunolocation of the TN and FN immunostaining patterns in different areas of dental tissues also were carried out by two blinded calibrated examiners.

RESULTS

Data from histological evaluation for pulps capped with MTA or CH in the different periods of time are demonstrated in Table 2.

Morphological Analysis

MTA—At seven days, no response or a mild inflammatory response was observed. Focal new dentin deposition was noticed with the dentin barrier in an initial stage of formation (Figure 1A). After 70 days, dentin barriers were formed completely, obliterating the exposure area, and the odontoblast-like cells were underlining these barriers (Figure 2A).

CH—After seven days, a focal moderate inflammatory response was frequently observed in pulps capped with CH under the site of exposure. Initial reparative dentin deposition was noticed, and the odontoblast-like cells started their alignment under it (Figure 1D). In the 70-day group, there was no inflammatory response, and dentin barriers were obliterating the exposure site with odontoblast-like cells aligned subjacent to these barriers (Figure 2D).

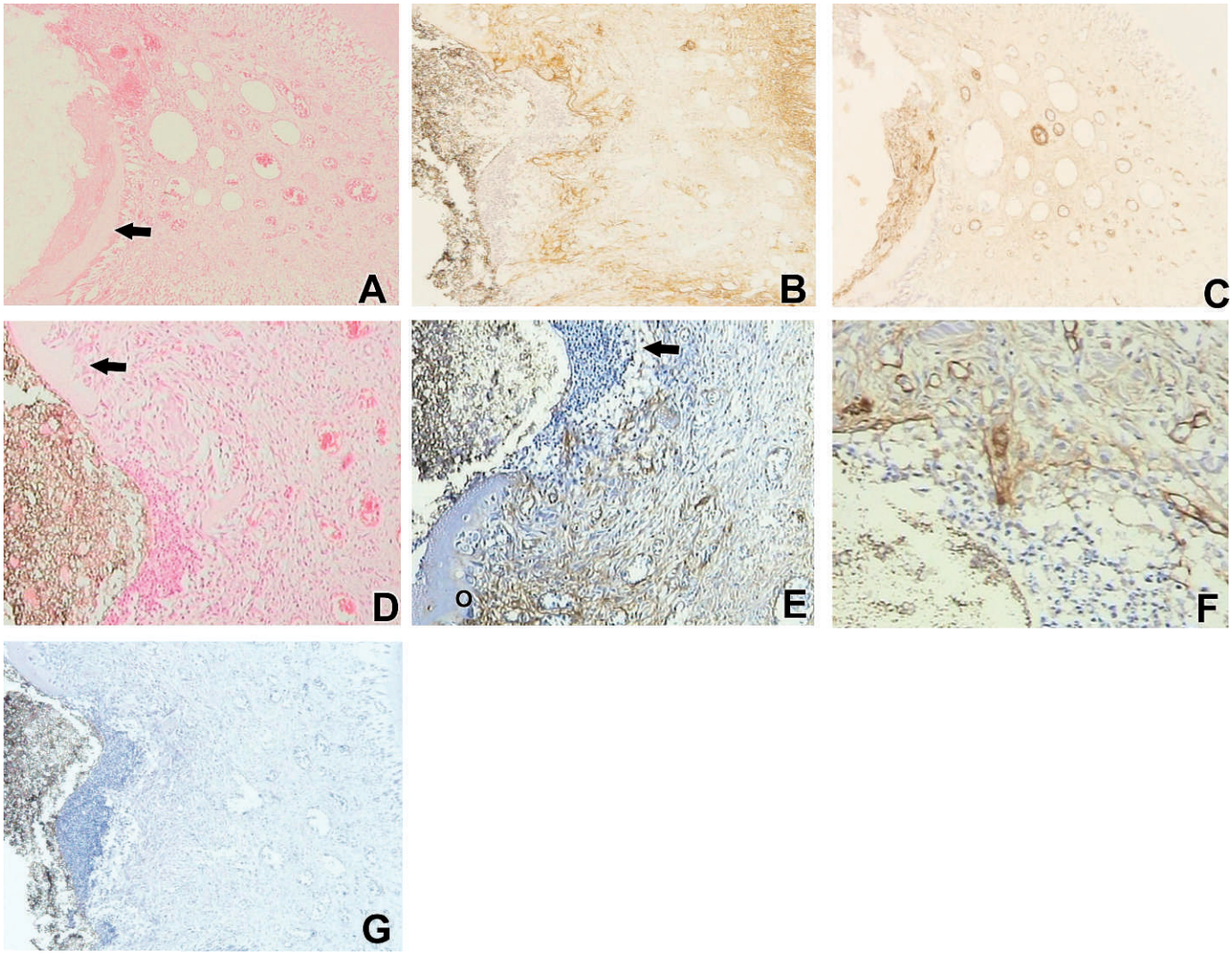

Figure 1. *Seven-day interval (A-C, treatment with MTA; D-F, treatment with CH). (A): No inflammatory response is observed, and an initial dentin deposition was noticed (arrow) (100×). (B): Immunoreactivity in reticular pattern for TN, observed in the entire pulp tissue, being more pronounced in the site of exposition (100×). (C): A positive staining was observed for FN in the entire pulp tissue, being also observed in the exposure site and around the blood vessels. Mineralized tissues (reactionary, reparative, and physiologic dentin) were not stained for FN (100×). (D): An inflammatory response was observed at the exposure site. An initial reparative dentin formation could be noticed, with some odontoblast-like cells positioned in this region (arrow; 100×). (E): There is no immunostaining for TN in the sites with inflammation (arrow). Positivity was also observed in the pulp tissue adjacent to the newly formed osteodentin barrier (O), which was negative for TN (100×). (F): No staining was observed in the exposure site where inflammatory infiltrate was present. FN shows a delicate fibrillar expression throughout the pulp tissue and also surrounding the blood vessels (200×).*

Statistical Results

A significantly higher inflammatory response was detected in the seven-day group capped with CH ($p<0.043$), and the inflammatory response decreased in the 70-day group. The two capping materials presented similar behaviors regarding soft-tissue organization. The postoperative time had a significant influence ($p<0.05$) on reparative dentin formation for both materials: CH (seven days)<CH (70 days; $p<0.001$) and MTA (seven days)<MTA (70 days; $p=0.004$). No significant difference was found between both materials when considering the reparative and reactionary dentin formation ($p>0.05$).

Immunohistochemical Analysis

The staining patterns for TN and FN were similar, independent of the capping material. At seven days, TN (Figure 1B,E) and FN (Figure 1C,F) showed similar patterns and immunolocation. They were observed under the exposure site and in the pulp tissue along collagen fibers and around blood vessels,

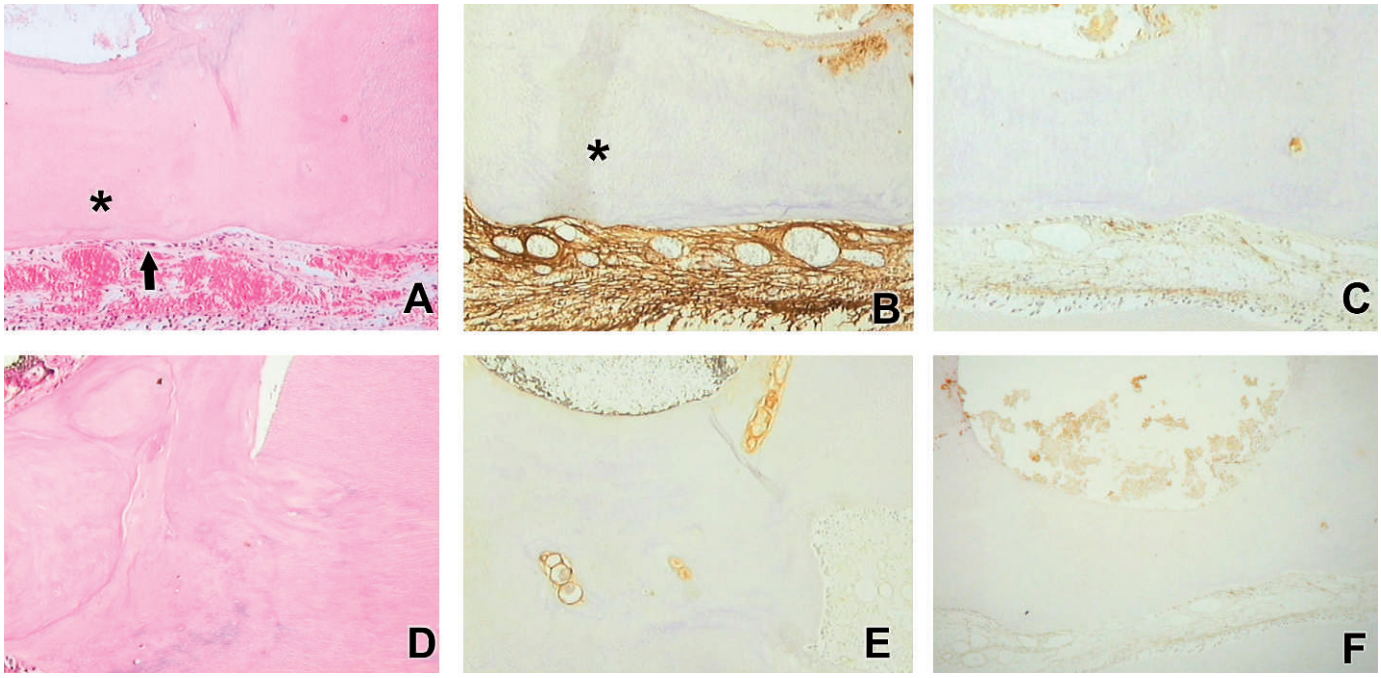

Figure 2. *Seventy-day interval (A-C, treatment with MTA; D-F, treatment with HC). (A): A dentin barrier was obliterating the exposure site, which was composed of an outer fibrodentin layer and an inner more organized dentin layer (*), with subjacent odontoblast-like cells aligned (arrow; 100×). (B): TN was detected throughout the pulp tissue and absent in the dentin barrier (*; 100×). (C): There was no immunostaining at the mineralized tissues, and discrete FN expression in a reticular pattern was noted (100×). (D): Intense mineralization was observed under the exposition area and subjacent to the pulp tissue. There was a tubular organization of this newly formed dentin (100×). (E): The mineralized tissue was negative for TN, and positive staining was observed in the pulp tissue (100×). (F): Weak and delicate staining for FN in the pulp tissue. Positive staining for blood vessels was also noted (internal controls). No staining was observed in the mineralized barrier (100×).*

demonstrating a reticular and well-defined pattern. In the presence of inflammatory infiltrate, there was no FN or TN expression. No staining was observed in newly formed reparative dentin for TN (Figure 1E) or FN, but the expression was detected in the underlining pulp tissue.

At 70 days, regarding the MTA and CH treatments, the dentin barriers formed were not positive for TN (Figure 2B,E) or FN (Figure 2C,F); however, the subjacent pulp tissue was positive for both (TN, Figure 2B,E; FN, Figure 2C,F), with a stronger expression for TN in the MTA group. There was immunoreactivity in the predentin area for TN (Figure 2B), while FN could be frequently absent or weakly expressed (Figure 2C). No immunoexpression for TN (Figure 2B,E) or FN (Figure 2C,F) was detected in dentin.

DISCUSSION

In this study, pig primary teeth with completely formed apexes were used to evaluate pulp repair. Animal models have been used to test pulp response,[20,21] but differences are observed between animal and human response.[5] Nevertheless, the use of human teeth is restricted by ethics concerns.[5,15,17] As a biological model, there are several similarities between swine and human beings, such as structure of internal organs, feeding pattern, enzymes, endocrine and immune system, and dental characteristics. In human primary and permanent teeth, the pulp development, formation, and structural histology are similar, and significant pulpal alterations are present only in the final stage of root resorption.[22] Also, a previous report used pig primary teeth to evaluate the histological response to CH or MTA.[9]

Using pig primary teeth as an animal model, Shayegan and others[9] compared as a direct pulp-capping agent different materials (beta tricalcium phosphate, white MTA, and white Portland Cement) to CH. The authors observed that all tested materials were biocompatible and had a similar performance to CH for producing pulpal healing, and the authors considered pig primary teeth a good model to perform direct capping studies.[9]

We observed less initial inflammatory response for MTA when compared with CH.[7] The MTA has shown a lower toxicity in primary teeth[23] and a higher metabolic activity in odontoblast-like cells as com-

pared with CH.[24] In addition, the necrosis produced by MTA is negligible.[7]

At 7 days, differences were observed in the expression of TN and FN between MTA and CH, when higher inflammatory response was present for CH. In inflamed areas, there is no staining for TN and FN[25] because of the destruction of the collagen fibers and other components of the extracellular matrix by the chemical mediators and proteolytic enzymes secreted by the inflammatory cells.[26] In noninflamed pulp tissue, TN and FN were accompanying the collagen fibers in the entire pulp tissue.[15,25] FN and TN were also noticed under the exposure site and adjacent to the initial dentin deposition. In the interodontoblastic region, a positive staining was observed for these two glycoproteins, as previously reported.[14,15,27] Fibrilar material has been reported between odontoblasts, corresponding to the von Korff fibers,[14] which corresponded to the positive FN fibers, glycoproteins that have an important role in keeping the elongated morphology of the odontoblasts.[14] During dental development, TN and FN are expressed in the basal membrane, and it is believed that they are enrolled in alignment and differentiation of dental papilla cells in odontoblasts in the presence of growth factors.[15] During pulp repair, it has been demonstrated that an FN and fibrin network in contact with calcium crystals could rmediate the migration, proliferation, and adhesion of cells that synthesize a fibrodentin matrix rich in FN, contributing to the odontoblast-like cells' differentiation.[14]

After 70 days, a favorable histological response was observed for both materials with normal tissue features, increased reactionary dentin deposition, as a result of upregulation of primary odontoblasts[17,18] and the deposition of reparative dentin, formed by odontoblast-like cells originated from dental pulp stem cells that undergo differentiation toward an odontoblast phenotype.[17,19] The reparative dentin formed was almost obliterating the region under the exposure site, forming a dentin barrier, which was composed of an outer osteodentin (fibrodentin) layer and an inner, more organized dentin layer.[3,5,17] Underlining this newly formed dentin, elongated odontoblast-like cells could be seen, as would be expected in this time interval.[5,17] Considering TN and FN expressions at this later period, no immunostaining was observed for either glycoprotein in dentin structures.[15] TN is expressed in extracellular matrices undergoing mineralization and is absent when these matrices complete their mineralization.[23] Predentin is considered a front of mineralization, and TN had a strong immunoreactivity in this region.[15,25]

The solubilization of dentin by CH and MTA releases bioactive components, sequestered into dentin matrix, signaling gene expression in pulp cells, which mediates the cell behavior observed during regeneration.[11,12] Calcium ions can stimulate the FN gene expression in pulp cells,[28] inducing the differentiation of pulp cells in mineralized tissue forming cells. TN was upregulated when dental pulp stem cells were treated with dentin matrix components during differentiation in odontoblast-like cells.[29]

Immunohistochemistry studies of a variety of tissues, in normal or altered functions, have shown that the distribution of TN is spatially and chronologically restricted. These findings suggest that TN probably functions as a homeostatic factor in the repair of tissue perturbation.[30]

Here we observed healing when CH and MTA were used as capping agents in primary pulps. There is a lack of histological studies in primary pulps, and generally, following exposition, a pulpotomy is the preferred treatment choice.[31] Our findings demonstrated that primary pulps are able to repair when capped with CH or MTA after traumatic injury by perforation, corroborating a previous study.[9] Primary pulps may have a higher regenerative capacity than previously demonstrated,[31] and the presence of stem cells in the pulp tissue could be the main reason for this ability.[32]

It is critical to highlight the importance of understanding the role of dentinal and pulp extracellular matrices during reparative events.[13] This will help to develop regenerative approaches in future applications of dental pulp tissue engineering.[32,33]

CONCLUSIONS

Overall, the two capping materials produced similar results in pig primary teeth. Little inflammatory response was detected for MTA in the initial period as compared with CH; however, pulp repair demonstrated as dentin barrier formation was equally noted in pulps capped with both materials. FN and TN expression were similar for both capping materials, and they were expressed throughout the reparative events but not during the inflammatory events.

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