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Tricalcium Silicate Capping Materials Modulate Pulp Healing and Inflammatory Activity *In Vitro*

Thomas Giraud, MSc,^{*†} Charlotte Jeanneau, PhD,^{*} Madison Bergmann, MSc,^{*} Patrick Laurent, PhD,^{*†} and Imad About, PhD^{*}

Abstract

Introduction: On stimulation by lipoteichoic acid or by a physical injury, fibroblasts have been shown to play a major role in the initiation of the pulp inflammatory reaction and healing through secretion of complement proteins and growth factors. The application of direct pulp-capping materials on these cells may interfere with the inflammatory and the healing processes within the pulp's inextensible environment. This work was designed to study *in vitro* the effects of silicate-based materials on pulp fibroblast modulation of the initial steps of pulp inflammation and healing. **Methods:** The effects of Biodentine, TheraCal, and Xeno III eluates were studied on lipoteichoic acid-stimulated and physically injured fibroblasts. Cytokine secretion (interleukin 6, vascular endothelial growth factor, fibroblast growth factor-2, and transforming growth factor- β 1) was quantified by enzyme-linked immunosorbent assay. Inflammatory THP-1 adhesion to endothelial cells and their migration and activation were studied *in vitro*. Human pulp fibroblast proliferation was investigated with the MTT test, and their migration to the injury site was studied with the scratch healing assay. **Results:** Interleukin 6 and vascular endothelial growth factor secretion increased with all materials but to a lesser extent with Biodentine. Fibroblast growth factor-2 and transforming growth factor- β 1 secretion was significantly higher with Biodentine than with all other materials. THP-1 cell adhesion to endothelial cells and their activation were reduced by Biodentine and TheraCal. However, their migration decreased only with Biodentine. Fibroblast proliferation significantly increased with Biodentine but significantly decreased with Xeno III after day 6. Finally, only Biodentine induced fibroblast migration to the injury site in the scratch assay. **Conclusions:** These results confirm that pulp-capping materials affect the early steps of pulp inflammation and healing. They show that Biodentine had the highest pulp healing and anti-inflammatory potential when compared with the resin-containing materials. This highlights the interest of the material choice for direct pulp-capping. (*J Endod* 2018; ■:1–6)

Key Words

Pulp capping materials, pulp healing, pulp inflammation, tricalcium silicates

Deep carious or physical lesions may lead to dental pulp injuries. Subsequently, both pulp-infiltrating bacteria and injured tissues initiate an inflammatory reaction (1).

The inflammatory reaction implies secretion of cytokines that contribute to immune cell recruitment. These cells adhere on the vascular endothelium and transigrate and reach the injured tissue guided by a chemotaxis gradient to be finally activated as macrophage-like cells (2). Many cytokines such as interleukin (IL) 6, IL-8, and IL-1 β are involved in the inflammatory events. Others, such as vascular endothelial growth factor (VEGF), play a role both in the vasodilation of the vessels after the initiation of the inflammation and in pulp tissue vascularization during the regenerative processes after pulp hypoxia (3).

Pulp healing implies an initial inflammatory reaction followed by fibroblasts and dental pulp stem cell proliferation and migration to the injury site to replace the lost tissue. It has been well-demonstrated that many of these events are locally regulated. Many growth factors involved in these steps are either sequestered in the dentin and released because of the acidic environment during the carious progression or secreted by the underlying pulp cells (4–6). Among these, transforming growth factor β 1 (TGF- β 1) has been shown to play a key role in dental pulp stem cell migration and differentiation (7, 8), whereas fibroblast growth factor 2 (FGF-2) has been shown to be involved in dentin-pulp complex regeneration inducing pulp cell proliferation and migration (9).

In addition, recent data have confirmed the involvement of the complement system in the balance between pulp inflammation and regeneration through secretion of active molecules such as C3a, C5a, and membrane attack complex within this inextensible environment (10–12). Furthermore, it has been shown that pulp-capping material can modulate C5a secretion (13).

Tricalcium silicates are now considered as the materials of choice for vital pulp therapy because histologic studies have demonstrated dentin-pulp regeneration without pulp inflammation *in vivo* (14) and in human teeth (15, 16). Modifications of these types of materials aiming at reducing their setting time led to the development of light-cured tricalcium silicates such as TheraCal (Bisco, Schaumburg, IL). However,

Significance

Direct pulp-capping materials modulate pulp inflammation and healing. Whereas tricalcium silicates shift the balance toward healing, resin-containing materials drive it toward inflammation. Thus, within the limits of this study, resin-containing materials cannot be recommended for direct pulp-capping.

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the presence of a high percentage of resins in this material raises questions about its possible effects on the pulp inflammatory reaction because of its reported toxicity (16, 17).

The therapeutic use of these materials in direct pulp-capping implies their direct application on the underlying fibroblasts that have been reported to play a significant role in initiating the pulp inflammatory response and regeneration. Indeed, these cells express and secrete complement active proteins such as C3a and C5a, known as anaphylatoxins, which induce vascular modifications and recruitment of inflammatory cells during the inflammatory reaction (18) and the recruitment of pulp stem cells during the regeneration (12, 19). Pulp fibroblasts also secrete VEGF, platelet-derived growth factor, and FGF-2, which directly affect pulp neovascularization. Previous studies on cell injury or incubation with lipoteichoic acid (LTA), a component of gram-positive bacteria, demonstrated a significant increase in the secretion of these molecules. This demonstrates a link between fibroblast stimulation and the initiation of the inflammatory reaction or regeneration process (4, 20, 21).

The aim of this *in vitro* study was to determine the influence of tricalcium silicate-based capping materials on pulp fibroblast stimulation and involvement in the initial steps of pulp inflammation and healing and to investigate the consequences of adding resins on these events.

Although Biodentine (Septodont, Saint-Maur-des-Fossés, France) and TheraCal are tricalcium silicate-based materials, TheraCal also contains resins to allow its light-curing. Xeno III (Dentsply Sirona, York, PA) is a resin bonding system used as a control. Eluates of the above mentioned materials were applied on LTA-stimulated and physically injured fibroblasts. We evaluated fibroblast secretion of cytokines involved in inflammation (IL-6, VEGF) and regeneration (TGF- β 1, FGF-2). We also studied inflammatory cell adhesion on endothelial cells, their migration, and activation. Finally, we investigated fibroblast proliferation and colonization of the injury area.

Materials and Methods

Reagents

Media, reagents, and cell culture supplies were from Dutscher (Brumath, France).

Human Pulp Fibroblast Culture

Human primary pulp cells were prepared from immature third molars extracted for orthodontic reasons (male and female patients younger than 18 years) in compliance with French legislation (informed patients consent and institutional review board approval of the protocol used) by the explant outgrowth method as described previously (22). After sorting with the STRO-1 mesenchymal stem cell marker (12), pulp stem cells were removed. The remaining STRO-1 negative cell population was characterized as fibroblasts. Briefly, they were positive for fibroblast surface protein and negative for Nanog, Oct3/4, Sox 2, Klf 4, CD 44, CD90, CD 105, CD 146, and CD 166 (10, 21). These fibroblasts were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B and used in this study.

Conditioned Medium Preparation

The materials were prepared according to manufacturers' instructions and inserted into standardized silicone molds. Samples of set materials were sterilized with UV (20 minutes on each side) and incubated in serum-free MEM (0.05 cm²/mL) for 24 hours at 37°C, 5% CO₂ as described previously (13). These media will hereafter be called conditioned media. MEM culture medium was used as a control.

Fibroblast Treatment

To simulate bacterial infection in caries lesions, confluent fibroblast cultures (30,000 cells/cm²) were stimulated with 1 μ g/mL LTA for 4 hours. In addition, physical injuries were performed to simulate the cell injury during the cavity preparation process. This was done with sterile scalpels in vertical and horizontal directions (10 in each direction in 6-well plates and 5 on each in 12-well plates) in serum-free MEM. For clarity, these LTA-stimulated and injured fibroblasts will be hereafter referred to as treated fibroblasts.

Conditioned Medium Toxicity

Treated fibroblasts were incubated with the conditioned or MEM control medium. After 4, 24, and 48 hours the supernatants were removed, and MTT test (Sigma-Aldrich, St Louis, MO) was performed as described previously (8) with the MTT substrate (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Cytokine Secretion by Treated Fibroblasts

Treated fibroblasts were cultured in conditioned or MEM control medium in 12-well plates. After 4, 24, and 48 hours, supernatants were used for cytokine (IL-6, VEGF, TGF- β 1, or FGF-2) quantification by the enzyme-linked immunosorbent assay using DuoSet kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Results are expressed in pg/mL.

Culture of Human Umbilical Vein Endothelial Cells and Inflammatory THP-1 Cells

Human umbilical vein endothelial cells (HUVECs) (PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium 2 (PromoCell). THP-1 cells, a human monocytic cell line (Sigma, St Quentin Fallavier, France), were cultured in RPMI (supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate), as described (23).

THP-1 Cell Recruitment Sequence

THP-1 Cell Adhesion on Endothelial Cells. Confluent HUVECs (80,000 cells/cm²) were cultured for 4 hours in 6-well plates with supernatants from treated fibroblasts cultured in conditioned or MEM control medium for 24 hours. THP-1 cells were first incubated with 0.1 μ mol/L BCECF acid (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) for 1 hour. Suspension of BCECF labeled THP-1 (25,000 cells/mL) was applied on the HUVEC monolayer for 30 minutes under gentle orbital agitation. Media were then removed, and HUVEC monolayers were washed 4 times with phosphate-buffered saline with calcium and magnesium (PBS). The THP-1 adherent cells were counted in 5 random fields by using a fluorescent microscope (\times 100). Results are expressed as percentage of the control.

THP-1 Cell Migration. Migration assays were performed in Boyden chambers. Treated fibroblasts were plated in the lower chambers (12-well plates) with 1 mL conditioned or MEM control medium. The upper chambers were seeded with 25,000 THP-1 cells in 100 μ L. THP-1 cells were activated with phorbol myristate acetate (400 ng/mL, 24 hours) before migration. After 24 hours, migrating cells on the lower side of the insert membrane were fixed (15 minutes, cold ethanol 70%) and stained with eosin (20 minutes). Migrating cells were counted in 5 random fields by using light microscopy (\times 100). Results are expressed as percentage of control.

THP-1 Cell Activation. THP-1 suspension (25,000 cells/mL) was cultured 24 hours in 12-well plates with supernatants from treated

fibroblasts cultured for 24 hours in conditioned or MEM control medium. Then, the wells were washed 4 times with PBS. This ensures removal of dead cells and allows for adhesion of living THP-1 cells only. Adherent cell nuclei were stained (20 minutes) with DAPI (4',6-diamidino-2-phenylindole), and the number of activated (adherent) cells was counted in 5 random fields by using a fluorescent microscope ($\times 100$). Results are expressed as percentage of the control.

Human Pulp Fibroblast Proliferation

Pulp fibroblasts were cultured at a low density (1000 cells/cm²) in 12-well plates for 24 hours. After this period, media were replaced by supernatants from treated fibroblasts cultured for 24 hours with conditioned or MEM control medium. After 3, 6, and 9 days the supernatants were removed, and a MTT test was performed to measure cell proliferation (24). Results are expressed as percentage of control at day 3 considered as the baseline.

Fibroblasts Scratch Wound Healing Assays

Confluent fibroblasts (30,000 cells/cm²) in 6-well plates were injured with a pipette tip and incubated with supernatants from treated fibroblasts cultured 24 hours with conditioned or MEM control medium. After 12 hours of incubation, cell nuclei were stained with 1 μ g/mL DAPI (20 minutes), and migrating cells to the injured zone were counted in 5 random fields under a fluorescence microscope ($\times 100$). Results are expressed as percentage of control.

Statistical Analysis

All experiments were done in triplicate and repeated 3 times. Data are expressed as mean \pm standard error of mean. Student *t* test was used to determine statistical significance ($P < .05$).

Results

Pulp-Capping Materials Conditioned Media Were Not Toxic to Treated Fibroblasts

There was no statistically significant difference in the viability of treated fibroblasts cultured with pulp-capping materials conditioned media compared with the control regardless of the incubation time (data not shown).

Biomaterials Modified Cytokine Secretion

Applying all biomaterial conditioned media on treated fibroblasts led to a significant increase of IL-6 secretion. However, this increase was significantly lower with Biodentine regardless of the incubation period (Fig. 1A). VEGF was secreted in all culture conditions. This secretion increased with all materials but was significantly higher with resin-containing materials (TheraCal and Xeno III) (Fig. 1B). Biodentine induced a significantly higher secretion of TGF- β 1 after 24 and 48 hours, whereas a decrease was observed with TheraCal and Xeno III after 24 hours (Fig. 1C). Finally, a rapid increase in FGF-2 secretion was observed after 4 hours with all materials. However, a significantly higher

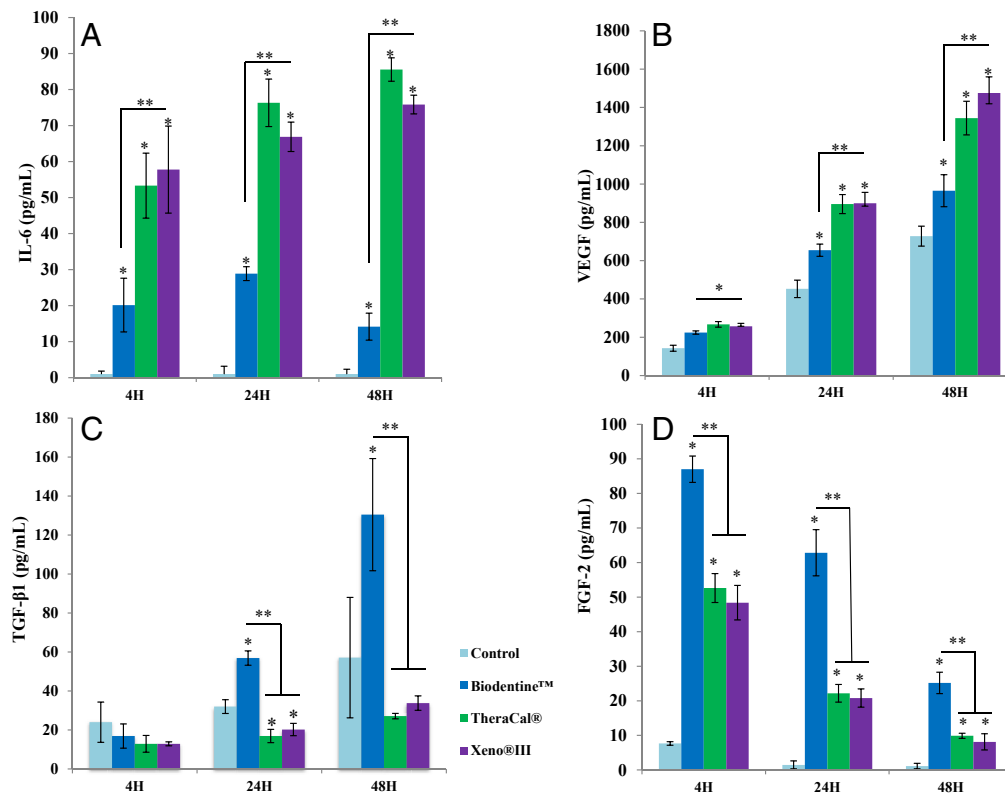


Figure 1. Effects of biomaterials on cytokine secretion. (A) IL-6 secretion by treated fibroblasts significantly increased with all biomaterials conditioned media regardless of incubation time. This secretion was significantly lower with Biodentine. (B) All incubation conditions led to significant increase of VEGF secretion. After 24 and 48 hours, the secretion was statistically higher in all conditioned media compared with the control but to a lesser extent with Biodentine. (C) Treated fibroblasts secreted significantly more TGF- β 1 after 24 and 48 hours only when incubated with Biodentine. (D) Treated fibroblasts incubated with all conditioned media showed significant increase in FGF-2 compared with the control, but this level was higher with Biodentine compared with TheraCal and Xeno III. Results are expressed in pg/mL. *Significant difference as compared with the control; **significant differences between 2 conditions ($P < .05$).

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level was obtained with Biodentine as compared with TheraCal and Xeno III regardless of the incubation period (Fig. 1D).

Inflammatory Cell Recruitment Modulation

Inflammatory THP-1 cell adhesion on HUVEC monolayer assay protocol is represented (Fig. 2A) and illustrated with representative pictures used for cell counts (Fig. 2Ba–d). THP-1 cell adhesion significantly decreased when the HUVECs were cultured with Biodentine and TheraCal conditioned media. Adhesion with Biodentine was significantly lower than with TheraCal. By contrast, Xeno III significantly increased this adhesion as compared with the control and with the other materials (Fig. 2C). THP-1 cell migration significantly decreased only with Biodentine conditioned medium (Fig. 3A). Activation of THP-1 cells representative pictures were presented (Fig. 3Ba–d) and used for cell count. This activation decreased with silicates-containing materials conditioned media (Biodentine and TheraCal), whereas it increased with Xeno III (Fig. 3Be).

Materials Modulate Human Pulp Fibroblast Proliferation and Migration

Biodentine conditioned medium significantly increased fibroblast proliferation at all incubation periods (Fig. 4A) and significantly increased fibroblast colonization of the injury area (Fig. 4Ba–e). By contrast, Xeno III decreased fibroblast proliferation at 6 and 9 days, whereas TheraCal significantly increased fibroblast proliferation only at 6 days but to a lesser extent than Biodentine (Fig. 4A). Finally, TheraCal and Xeno III did not affect fibroblast colonization of the injury area (Fig. 4Ba–e).

Discussion

This work shows that after simulating pulp fibroblast injury and bacterial infection, applying pulp-capping material eluates on these cells affects pulp inflammation and healing processes. Both events are crucial because their balance is a pre-requisite for the regeneration process within the pulp's inextensible environment. This study shows that although tricalcium silicates shift the balance toward the healing process, resin-containing materials drive this balance toward an inflammatory reaction.

The influence of these materials on pulp fibroblast response was investigated on LTA-stimulated and injured fibroblasts. This simulated some of the clinical aspects of pulp-capping procedure that is performed after carious decays on pulps subjected to injuries with dental burs during the cavity preparation. The physical injury protocol has been reported in multiple studies in different tissues using pipette tips or scalpels (25, 26). Because the majority of bacterial species involved in the cariogenic process are gram positive, the presence of these bacteria is simulated in this work by incubating the fibroblasts with LTA (11, 21, 27).

Here, materials were already set and were not placed in direct contact with the cells. Instead, eluates of these materials were applied on the cells. Indeed, a necrotic zone is formed *in vivo* because of the alkaline pH of calcium hydroxide or tricalcium silicate-based materials (28, 29), whereas only eluates of set capping materials reach the underlying viable cells.

The proinflammatory activity was studied here by investigating the effects of these materials on the pulp fibroblasts in the early steps of the inflammation including secretion of proinflammatory cytokines, adhesion of inflammatory THP-1 cells on blood vessel endothelial cells, their migration, and activation at the injury site. Indeed, although injured cells initiate the inflammatory reaction by secreting proinflammatory cytokines, our study demonstrates that applying pulp-capping

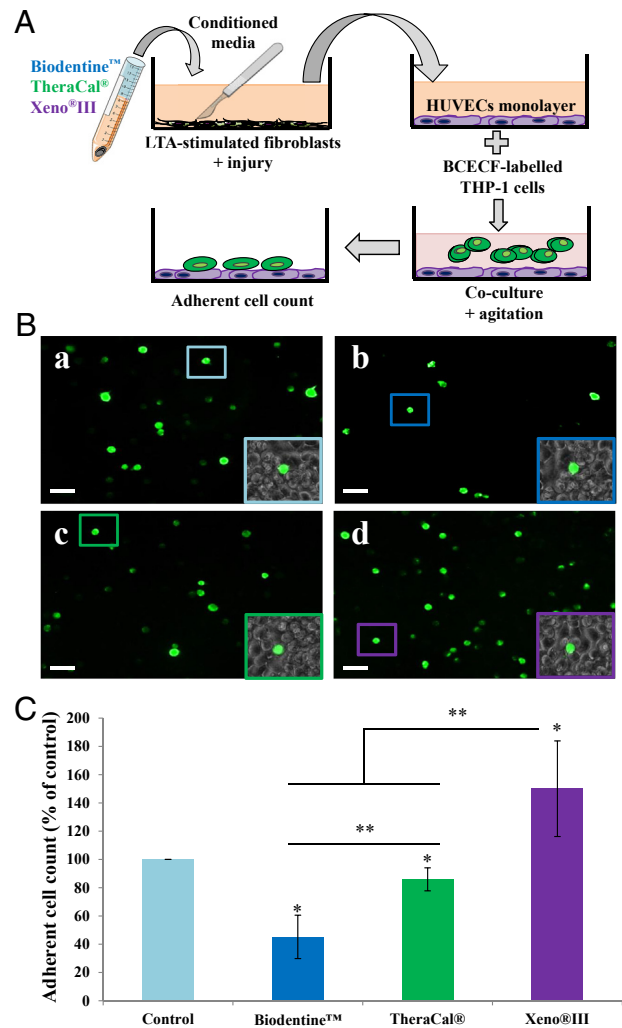


Figure 2. Inflammatory THP-1 adhesion on endothelial cells (HUVECs). (A) Schematic representation of THP-1 cell adhesion on HUVEC assay. A monolayer of HUVECs was stimulated for 4 hours with supernatants from treated fibroblasts cultured 24 hours with conditioned or MEM control media. In parallel, THP-1 cells were labelled with BCECF acid for 1 hour. Both cells were then co-cultured for 30 minutes under gentle agitation to avoid spontaneous cell attachment. After this incubation time, media were removed, and adherent THP-1 cells to the endothelial cell layer were counted with fluorescent microscope ($\times 100$). (B) Representative pictures on fluorescent microscopy of the adhesion assay showing (a) control, (b) Biodentine, (c) TheraCal, and (d) Xeno III. Framed areas show adherent fluorescent THP-1 cells on endothelial cell monolayer. Scale bars, 50 μm . (C) Quantification of THP-1 adhesion on endothelial cells. Biodentine and TheraCal significantly decreased THP-1 cell adhesion on HUVECs compared with the control and with Xeno III, which induced significantly more adhesion. Number of adhering cells with Biodentine was significantly lower than with TheraCal. Results are expressed in percentage of the control. *Significant differences as compared with the control; **significant differences between 2 conditions ($P < .05$).

materials conditioned media on these cells modulate cytokine secretion. All materials induced a significant release of IL-6 and VEGF. IL-6 is considered a potent cytokine playing a major role in the acute inflammation process (30), and VEGF is known to increase vascular permeability (31). This allows inflammatory cells to adhere to blood vessel endothelial cells and their migration to the injury site where these cells are activated.

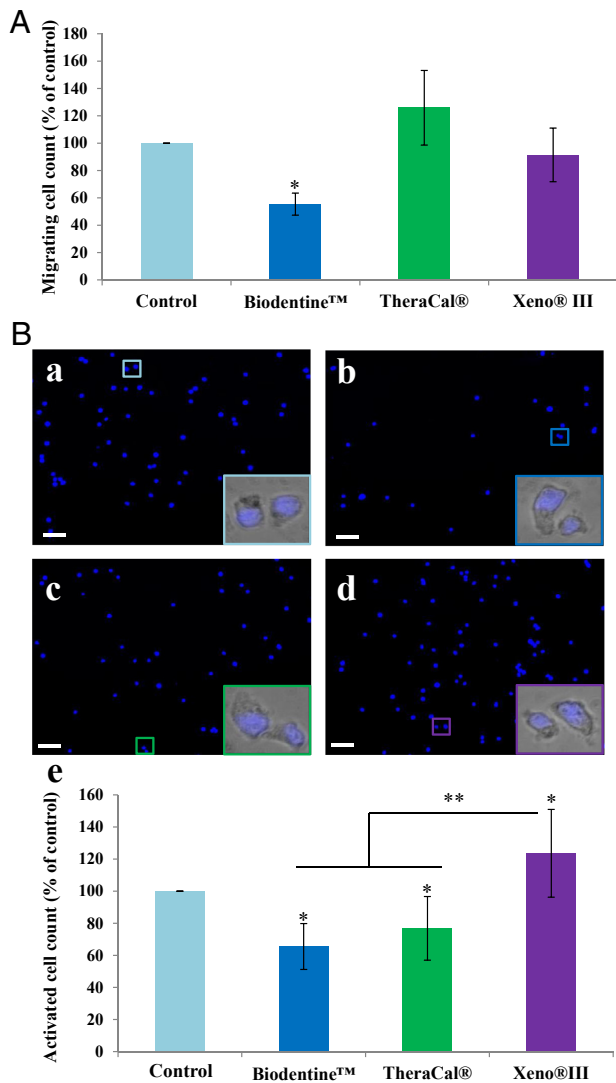


Figure 3. THP-1 cell migration and activation. (A) THP-1 cell migration assay in Boyden chambers showed that Biodentine significantly reduced this migration, which was not affected by TheraCal or Xeno III. (B) THP-1 activation assay. THP-1 cells were stimulated for 24 hours with supernatants from treated fibroblasts incubated with conditioned or MEM control media for 24 hours. (a–d) Representative pictures on fluorescent microscopy showing (a) control, (b) Biodentine, (c) TheraCal, and (d) Xeno III. Framed areas show superimposition phase-contrast and fluorescent images of adherent cells, indicating their activation into macrophage-like cells. Scale bars, 200 μm . (e) THP-1 cell activation was significantly reduced by TheraCal and Biodentine and significantly increased with Xeno III. Results are expressed in percentage of control. *Significant difference as compared with the control; **significant differences between 2 conditions ($P < .05$).

The secretion level of IL-6 and VEGF was significantly higher with resin-containing materials than with Biodentine. This is confirmed by the fact that Xeno III significantly induced THP-1 adhesion to endothelial cells and their activation. This reflects a stimulation of the inflammatory reaction and confirms the resin-containing materials' inflammatory potential when applied directly to the dentin-pulp complex. By contrast, our results show that the secretion level of proinflammatory IL-6 and VEGF with Biodentine was significantly lower than that with resin-containing materials. This can be related to the significant decrease of THP-1 adhesion to endothelial cells and their decreased migration

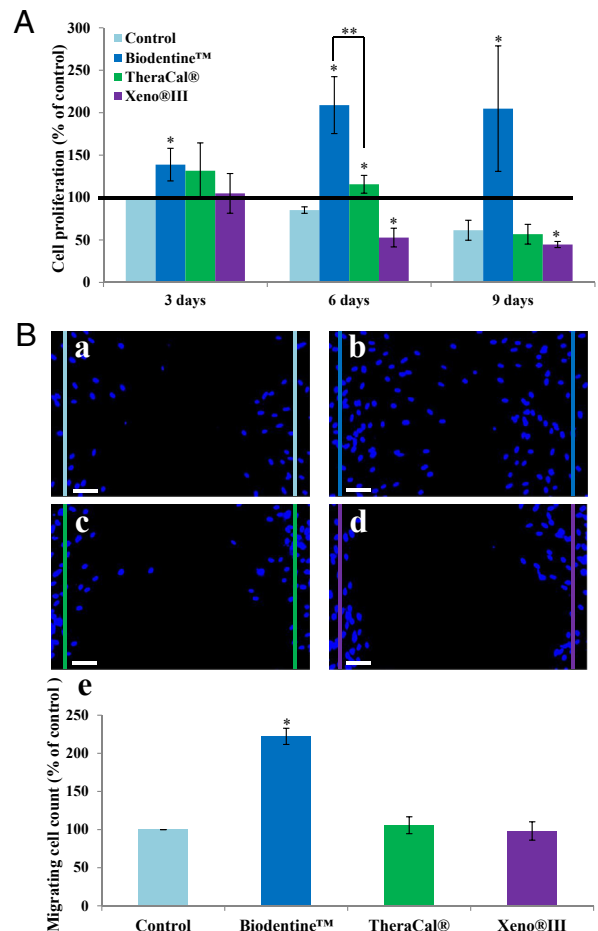


Figure 4. Human pulp fibroblast proliferation and scratch wound healing assays. (A) Pulp fibroblast proliferation. Biodentine increased fibroblast proliferation at all incubation periods. An increase in fibroblast proliferation was observed with TheraCal at 6 days but to a lesser extent than with Biodentine. Xeno III decreased fibroblast proliferation after 6 and 9 days. (B) Scratch wound healing assays of fibroblasts cultured 12 hours with supernatants from treated fibroblasts. (a–d) Representative pictures taken with fluorescent microscope: (a) control, (b) Biodentine, (c) TheraCal, and (d) Xeno III. The 2 vertical lines delineate the area initially devoid of cells because of the injury preparation. Migrating fibroblasts to this area were counted under a fluorescent microscope. Scale bars, 200 μm . (e) Number of cells migrating to the injury area significantly increased only with Biodentine. Results are expressed in percentage of the control. *Significant differences with the control; **significant differences between 2 conditions ($P < .05$).

and activation. These results suggest an inflammatory inhibiting potential with Biodentine.

On the other hand, our study demonstrated that Biodentine increased FGF-2 and TGF- β 1 secretion. This can be correlated to the observed stimulation of fibroblast proliferation and the induction of their migration to the injury site in the scratch assay. By contrast, Xeno III decreased TGF- β 1 secretion and decreased fibroblast proliferation. This is in line with previous works that reported the involvement of FGF-2 and TGF- β 1 in the neoangiogenesis during the healing process (4) and their involvement in pulp fibroblast and stem cell proliferation and their recruitment (21).

TheraCal, which contains resins and silicates, increased the proinflammatory IL-6 and VEGF cytokine secretion as observed with Xeno III. But unlike Xeno III, TheraCal reduced inflammatory cell adhesion on endothelial cells and their activation but to a lesser extent than

Biodentine. TheraCal did not affect fibroblast proliferation (except after 6 days) and did not induce their migration. These results are very close to those obtained with Xeno III and suggest that at a cellular level, the addition of resins alters the bioactive silicate properties in the context of the pulp inflammation and healing balance.

Taken together, these results show that while Biodentine decreases the inflammation, it induces the secretion of growth factors involved in the healing process. These results are in line with previously published investigations on human tooth cultures where Biodentine induced a high mineralization potential in well-organized pulp tissue with increased expression of dentin sialoprotein and nestin, indicating odontoblastic differentiation and mineralization potential. By contrast, decreased expression of these markers was obtained with TheraCal with disorganized and scattered mineralization within the pulp tissue (16).

Moreover, this study provides an explanation of the consequences of adding resins to tricalcium silicates at the cellular level. It appears that resin toxicity initiates an inflammatory reaction within the dental pulp that is located within rigid dentinal walls. This inflammation may be deleterious to the pulp regeneration process. Indeed, partial pulpotomy of human teeth with TheraCal revealed pulp tissue disorganization without formation of an organized dentin bridge, whereas a well-organized and thick bridge was formed with Biodentine (32). The absence of mineralization may be due to the release of monomers that, in addition to their toxicity (33, 34), have been shown to inhibit pulp cell differentiation and mineralization (35).

Overall, this work highlights the fact that the choice of pulp-capping material is of prime importance in direct pulp-capping procedures. Indeed, increasing the inflammatory reaction in a closed environment may lead to pulp destruction and disorganization and inhibition of the healing and regeneration processes.

Thus, in spite of decreasing the setting time of resin-based tricalcium silicates by light-curing and within the limits of this work performed *in vitro*, our work does not support using resin-containing materials for direct pulp-capping. Instead, it shows that adding resins alters the bioactive properties of tricalcium silicates.

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The authors deny any conflicts of interest related to this study.

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