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# Light-cured Tricalcium Silicate Toxicity to the Dental Pulp

*Charlotte Jeanneau, PhD,* Patrick Laurent, PhD,*† Charlotte Rombouts, PhD,* Thomas Giraud, MD,*† and Imad About, PhD**

## Abstract

**Introduction:** Numerous studies reported dentin bridge formation after pulp capping with tricalcium silicates. By contrast, pulp capping with resins leads to pulp toxicity and inflammation. Hybrid materials made up of tricalcium silicates and resins have also been developed to be used in direct pulp capping. This work was designed to study the consequences of adding resins to tricalcium silicates by investigating TheraCal (BISCO, Lançon De Provence, France) and Biodentine (Septodont, Saint Maur des Fosses, France) interactions with the dental pulp. **Methods:** Media conditioned with the biomaterials were used to analyze pulp fibroblast proliferation using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test and proinflammatory cytokine interleukin 8 (IL-8) secretion using the enzyme-linked immunosorbent assay. The effects of conditioned media on dentin sialoprotein (DSP) and nestin expression by dental pulp stem cells (DPSCs) were investigated by immunofluorescence. The materials' interactions with the vital pulp were investigated using the entire tooth culture model. **Results:** TheraCal-conditioned media significantly decreased pulp fibroblast proliferation, whereas no effect was observed with Biodentine. When DPSCs were cultured with Biodentine-conditioned media, immunofluorescence showed an increased expression of DSP and nestin. This expression was lower with TheraCal, which significantly induced proinflammatory IL-8 release both in cultured fibroblasts and entire tooth cultures. This IL-8 secretion increase was not observed with Biodentine. Entire tooth culture histology showed a higher mineralization with Biodentine, whereas significant tissue disorganization was observed with TheraCal. **Conclusions:** Within the limits of these preclinical results, resin-containing TheraCal cannot be recommended for direct pulp capping. *(J Endod 2017;■:1–7)*

## Key Words

Entire tooth culture, pulp capping materials, resin, tricalcium silicates

### Significance

TheraCal is toxic to pulp fibroblasts and has a higher inflammatory effect and a lower bioactive potential than Biodentine. Within the limits of these preclinical results, TheraCal cannot be recommended for direct pulp capping.

Maintaining pulp vitality after carious, traumatic, or iatrogenic injuries remains a challenge in immature permanent teeth in which pulp vitality allows completion of root formation. Several biomaterials are used in vital pulp therapy, with the prognosis depending on several factors such as the material's biocompatibility, its effects on the inflammatory reaction, and its ability to prevent bacterial microleakage. The outcome also depends on the pulp ability to respond to injury (1).

Tricalcium silicate–based materials are raising more and more interest in the dental field. Among these, Biodentine, a recently developed calcium silicate–based dentin substitute, mainly composed of tricalcium silicates can be applied directly into the restorative cavity as a bioactive bulk dentin substitute and induces dentin bridge formation (2, 3). This bioactivity has been shown in entire tooth cultures *in vitro* (4) and in pulpotomy clinical trials (5) as well as in partial pulpotomy clinical case reports (6). TheraCal is a light-cured composite material made up of tricalcium silicate cement containing approximately 45% of resins. The resin consists of hydrophobic monomers, such as urethane dimethacrylate, bisphenol A-glycidyl methacrylate, and triethylene glycol dimethacrylate, and hydrophilic monomers, such as hydroxyethyl methacrylate. Unlike common dental composites, TheraCal contains polyethylene glycol dimethacrylate to allow the miscibility between the resin and the tricalcium silicate cement (7). The idea beyond developing this composite material is to take advantage of tricalcium silicate biocompatibility and well-demonstrated bioactivity by reducing its setting time (8). This light-cured composite material has been developed to be used as a direct pulp capping agent, in deep preparations, or as a protective liner/base for use under restorations with other materials. Experimental works performed *in vitro* reported a high toxicity of TheraCal to pulp cells (9). When applied directly onto the pulp in dog teeth *in vivo*, TheraCal induced an extensive pulp inflammatory reaction in 75% of the cases, whereas a complete dentinal bridge was formed only in 33% of the teeth (10).

Whenever the tooth dentin-pulp complex is affected by caries, attrition, erosion, chemicals, abrasion, and iatrogenic trauma, the pulpal response to these injuries can stimulate an inflammatory reaction (11). Similarly during restorative procedures, the inflammatory reaction is also the initial step of tissue healing. However, the most severe pulp tissue inflammation is not a direct result of the surgical techniques and materials used to restore tooth structure but because of the leakage and ensuing bacteria after these events (12). This is because the immune system triggers an inflammatory

response to limit tissue damage by eliminating bacteria and cell debris. Paradoxically, these inflammatory reactions can injure the pulp, which is surrounded by rigid and inextensible dentin walls, and lead to pulp necrosis in response to cavity restorations. This explains why the pulp consequences of restorative procedures are generally evaluated *in vivo* by investigating their toxicity and the local inflammatory response of the dental pulp.

Because the previously mentioned materials are indicated for direct pulp capping, it is important to check if their application would not alter pulp tissue organization or function. Although several studies reported Biodentine induction of dentin bridge formation, few clinical studies were performed on the clinical outcome of TheraCal applications and the consequences on the pulp tissue.

Thus, the aim of this work was to investigate the consequences of adding resin to tricalcium silicates on pulp regeneration. An *in vitro* experimental design was used to evaluate the material effects on pulp fibroblast proliferation and the secretion of proinflammatory cytokine interleukin 8 (IL-8). The effect of biomaterials on the engagement of dental pulp stem cells (DPSCs) into the odontoblast differentiation pathway was studied by evaluating dentin sialoprotein (DSP) and nestin expression by immunofluorescence. Finally, we investigated the consequence of TheraCal interaction with the vital pulp compared with Biodentine using the entire tooth culture model.

## Materials and Methods

### Reagents

Media, reagents, and cell culture supplies were from Dutscher (Brumath, France). Chemicals were from Carlo-Erba (Val-de-Reuil, France). Nestin, DSP, and mesenchymal stem cell marker (STRO-1) primary antibodies were respectively from Merck Millipore (Billerica, MA), Santa Cruz Biotechnology (Dallas, TX), and R&D Systems (Lille, France). Secondary antibodies and 4′,6-diamidino-2-phénylindole were provided by Life Technologies (Saint-Aubin, France).

### Primary Pulp Cell Culture

Human pulp cells were prepared from immature third molars extracted for orthodontic reasons in compliance with French legislation (patients' informed consent and institutional review board approval of the protocol used) by the explant outgrowth method as described previously (13).

### Magnetic Cell Sorting

STRO-1 pulp stem cells were directly sorted from pulp cell cultures at passages 1 to 3 with mouse antihuman STRO-1 immunoglobulin M coupled with immune magnetic beads according to the manufacturer's protocol (Dynal, Oslo, Norway) as described previously (14). Cells were characterized as described previously (15); STRO-1–sorted cells will be referred to as DPSCs, whereas STRO-1–negative cells will be referred to as pulp fibroblasts as described previously (16).

### Conditioned Media Preparation

Conditioned media were obtained as described previously (17). The contact surface between the material and the culture media volume was 0.05 and 0.5 $cm^2$/mL.

### Fibroblast Proliferation Assay

Pulp fibroblasts were cultured at a low density (1000 cells/$cm^2$) in 6-well culture plates for 24 hours before use. After this period, the medium was removed and replaced by the conditioned medium or minimum essential culture medium (MEM) as a control. After 3, 5, and 7 days, the supernatants were removed, and the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test was performed as described previously (16).

### IL-8 Secretion by Cultured Fibroblasts

Pulp fibroblasts were seeded in 6-well culture plates until confluence. They were then incubated in conditioned media (0.05 $cm^2$/mL) or MEM as a control. After 24 and 48 hours, the supernatants were used for IL-8 quantification by enzyme-linked immunosorbent assay (ELISA) using Duoset kits (R&D Systems) according to the manufacturer's instructions.

### Immunofluorescence Staining

DPSCs were grown in 8-well glass culture chambers to 50% confluency. Cells were then cultured in MEM or conditioned medium for 7 days. Cells were fixed with 70% ethanol for 15 minutes at −20°C, and nonspecific binding sites were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 hour. Cells were then incubated for 1 hour with primary antibody against DSP (5 $\mu$g/mL) or nestin (2 $\mu$g/mL) and the respective isotypes. After washing, cells were incubated 45 minutes with 2 $\mu$g/mL of secondary antibody Alexa 594 antimouse immunoglobulin G and 1 $\mu$g/mL 4′,6-diamidino-2-phénylindole counterstain.

### Direct Pulp Capping Using Human Entire Tooth Culture Model

Immature third molars ($N$ = 33) extracted for orthodontic reasons were obtained in compliance with French legislation (patients' informed consent and institutional review board approval of the protocol used) and prepared as described previously (18). Third molars were then randomly assigned to TheraCal ($n$ = 11), Biodentine ($n$ = 11), and control ($n$ = 11) groups. Biodentine and TheraCal, prepared according to manufacturers' instructions, were applied directly onto the pulp. A 2-mm-thick layer of TheraCal was placed in the cavity and polymerized for 20 seconds after each 1-mm increment. Cavities of the control group were filled with MEM. The teeth were kept in culture for 15 days as described previously (18). At 24 and 48 hours, cultured medium were collected for IL-SA8 quantification by ELISA as described in the previous section. Results were normalized and expressed as concentration in pg/mL/foramina surface area in $cm^2$. At the end of the culture period, all the teeth were prepared as described previously (18).

### Immunohistochemistry

Tooth sections were deparaffinized and rehydrated. Nestin and DSP staining were performed as described previously (18). Hematoxylin-eosin staining (Sigma-Aldrich, St Louis, MO) was performed on tooth sections according to the manufacturer's instructions. Finally, the slides were analyzed by an operator blinded to the groups under a light microscope and classified using the following criteria:

1. Mineralization potential
   a. High ≥20 foci located under the cavity
   b. Moderate ≤20 foci located under the cavity
   c. None = absence of foci
2. Pulp tissue organization and morphology
   a. Normal pulp morphology
   b. Disorganization of pulp beneath the cavity
   c. Complete pulp disorganization

### Statistical Analysis

Three independent experiments were performed, and each was done in triplicate. Data are expressed as means ± standard error of mean. The Student *t* test was used to determine statistical significance ($P < .05$) for proliferation and the ELISA assay. The Fisher exact test was used to determine statistical significance ($P < .05$) of the entire tooth culture model results.

## Results

### TheraCal Decreased Pulp Fibroblast Proliferation

A significant decrease in fibroblast proliferation was observed after 3, 5, and 7 days of contact with TheraCal-conditioned media at 0.05 $cm^2$/mL compared with that in contact with Biodentine and MEM ($P < .05$, $n = 3$) (Fig. 1*A*). Increasing the TheraCal surface area to 0.5 $cm^2$/mL led to a drastic decrease of fibroblast proliferation to 20% (Fig. 1*B*). There was no statistically significant difference in the proliferation of fibroblasts cultured in contact with Biodentine-conditioned media compared with the control regardless of the incubation time or contact surface area (Fig. 1*A* and *B*).

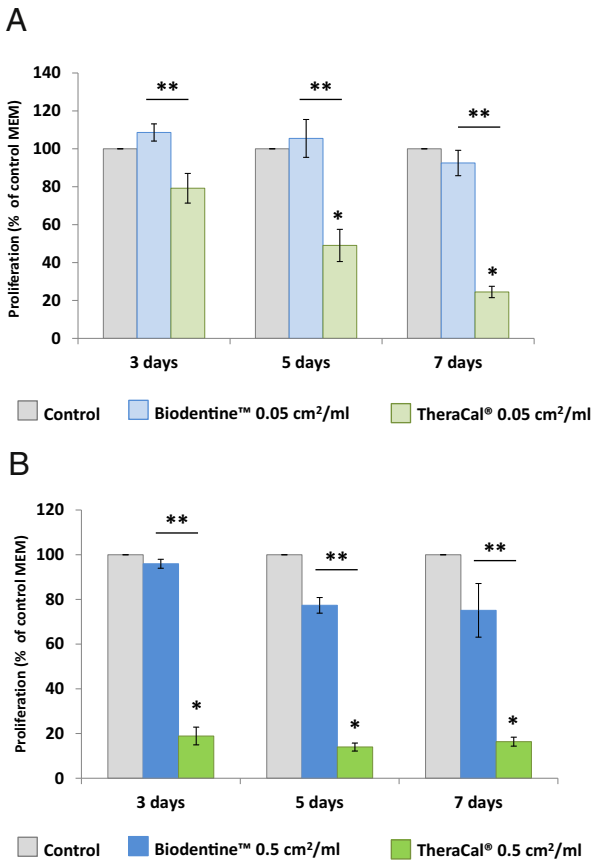



**Figure 1.** The effect of Biodentine and TheraCal on human pulp fibroblast proliferation. A significant decrease in pulp fibroblast proliferation was observed with TheraCal-conditioned media at (*A*) 0.05 $cm^2$/mL and (*B*) 0.5 $cm^2$/mL for all incubation periods. There was no statistically significant difference in fibroblast proliferation in contact with Biodentine-conditioned media compared with the control (MEM culture medium). The results are expressed as the mean cell proliferation ± standard deviation. *A significant difference compared with the control medium. **Significant differences between Biodentine and TheraCal conditions ($P < .05$).

### Biodentine Increased DPSC Expression of DSP and Nestin

DPSCs expressed a basal level of both DSP (Fig. 2*A*) and nestin (Fig. 2*B*). When DPSCs were cultured in contact with Biodentine-conditioned medium, an increased expression of both markers was observed (Fig. 2*C* and *D*). This increase was not observed with TheraCal-conditioned medium (Fig. 2*E* and *F*). No staining was detected in the negative controls (Fig. 2*G* and *H*).

### TheraCal Increased Fibroblast IL-8 Secretion

Applying Biodentine-conditioned media on pulp fibroblasts led to a significant increase of IL-8 release in the culture medium after 48 hours (Fig. 3*A*). However, when pulp cells were cultured with TheraCal-conditioned media, a significant increase of IL-8 secretion was observed after 24 and 48 hours. This increase was significantly higher than that obtained with Biodentine or the control (Fig. 3*A*).

### TheraCal Increased IL-8 Secretion by the Dental Pulp in Entire Teeth Cultures

IL-8 was secreted after 24 hours in the cultured media of all teeth (Fig. 3*B*). However, after 48 hours, a significant increase of IL-8 release was observed from the teeth with cavities filled with TheraCal compared with that from teeth with cavities filled with Biodentine or the control (Fig. 3*B*).

### Pulp Response after Capping with Biodentine and TheraCal in Entire Tooth Culture Model

As shown in Figure 4, when Biodentine was used as the pulp capping material, numerous mineralized foci were visible just beneath the material in the pulp that had a normal morphology (Fig. 4*Aa* and *Ab*). Few mineralization foci were observed beneath TheraCal in a disorganized pulp tissue (Fig. 4*Ae* and 4*Af*). In control teeth, no mineralized foci or pulp disorganization were observed, and the pulp had a normal morphology (Fig. 4*Ai* and 4*Aj*). Immunostaining revealed that nestin was expressed inside the foci, particularly in the sequestered cells with both materials (Fig. 4*Ac* and 4*Ag*). When Biodentine was used as a capping material, DSP was strongly expressed mostly at the periphery of the foci (Fig. 4*Ad*), whereas immunolabeling was weak or absent under TheraCal (Fig. 4*Ah*). No immunolabeling was observed in the negative controls (Fig. 4*Ak* and 4*Al*).

Analysis of 33 teeth revealed that there were statistically significant differences in the number of foci depending on the capping material used (Fig. 4*B*). A high or moderate number of foci was observed under Biodentine-filled cavities in 8 out of 11 teeth. In TheraCal-treated teeth, mineralization was absent in 5 teeth, whereas a moderate number of foci was observed in 6 out of 11 teeth (Fig. 4*B*). Pulp tissue organization in contact with Biodentine was normal or showing a local disorganization just beneath the material. By contrast, pulp tissue was partially or completely disorganized under TheraCal (Fig. 4*B*).

## Discussion

This work shows that TheraCal is toxic to pulp fibroblasts and has a higher inflammatory effect and a lower bioactive potential than Biodentine. This is demonstrated *in vitro* by investigating interactions between pulp fibroblasts or DPSCs and biomaterial-conditioned media and after direct pulp capping in a well-established human tooth culture model.

Investigating the interactions between the material-conditioned media and pulp fibroblasts showed that TheraCal significantly decreased pulp fibroblast proliferation. When the surface area with TheraCal increased from 0.05 to 0.5 $cm^2$/mL, a significant decrease

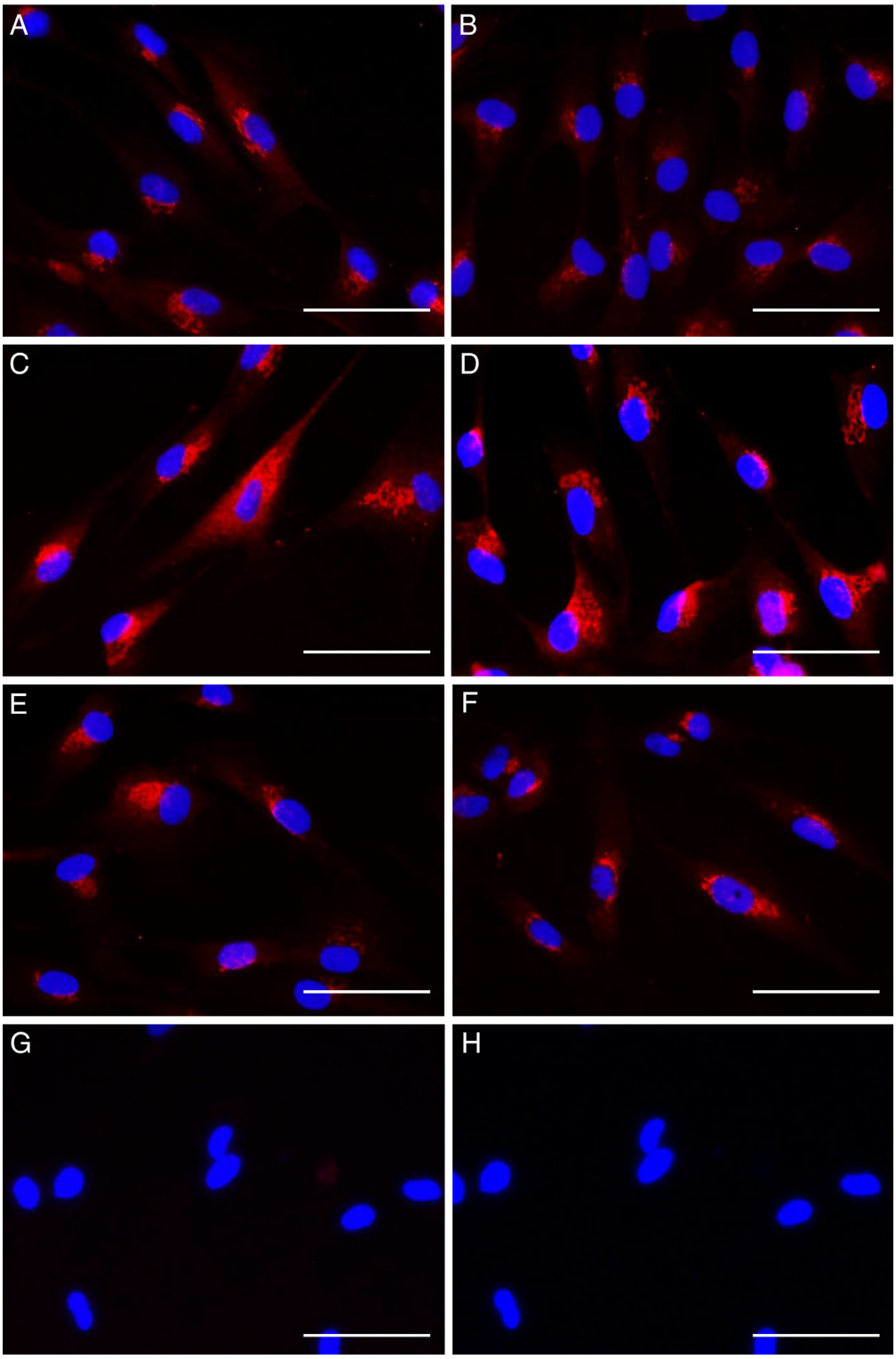



**Figure 2.** The effect of the capping biomaterials on DSP and nestin expression by DPSCs. DPSCs express a basal level of both (*A*) DSP and (*B*) nestin. An increase of both markers was observed when DPSCs were cultured in contact with (*C* and *D*) Biodentine-conditioned media but not with (*E* and *F*) TheraCal-conditioned media. (*G* and *H*) DSP and nestin staining was not detected in the negative controls when isotypes were used. Nuclei were counterstained with 4′,6-diamidino-2-phénylindole (*blue*). Scale bars = 50 $\mu$m.

in fibroblast proliferation was observed. This toxicity is in agreement with a previously published work showing that 31.5% of the cell metabolic activity was inhibited after a 24-hour contact period with TheraCal extract, and this inhibition reached 46% after 7 days (9). This may be caused by the fact that TheraCal contains resinous bisphenol A-glycidyl methacrylate, hydroxyethyl methacrylate, and urethane dimethacrylate

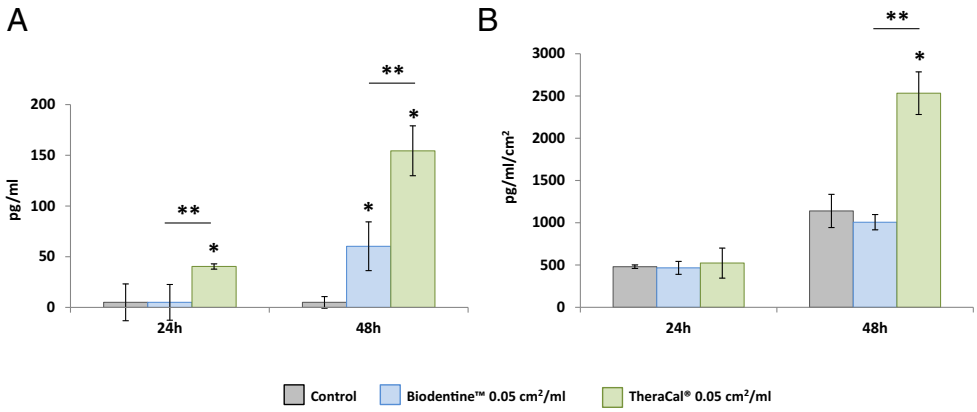


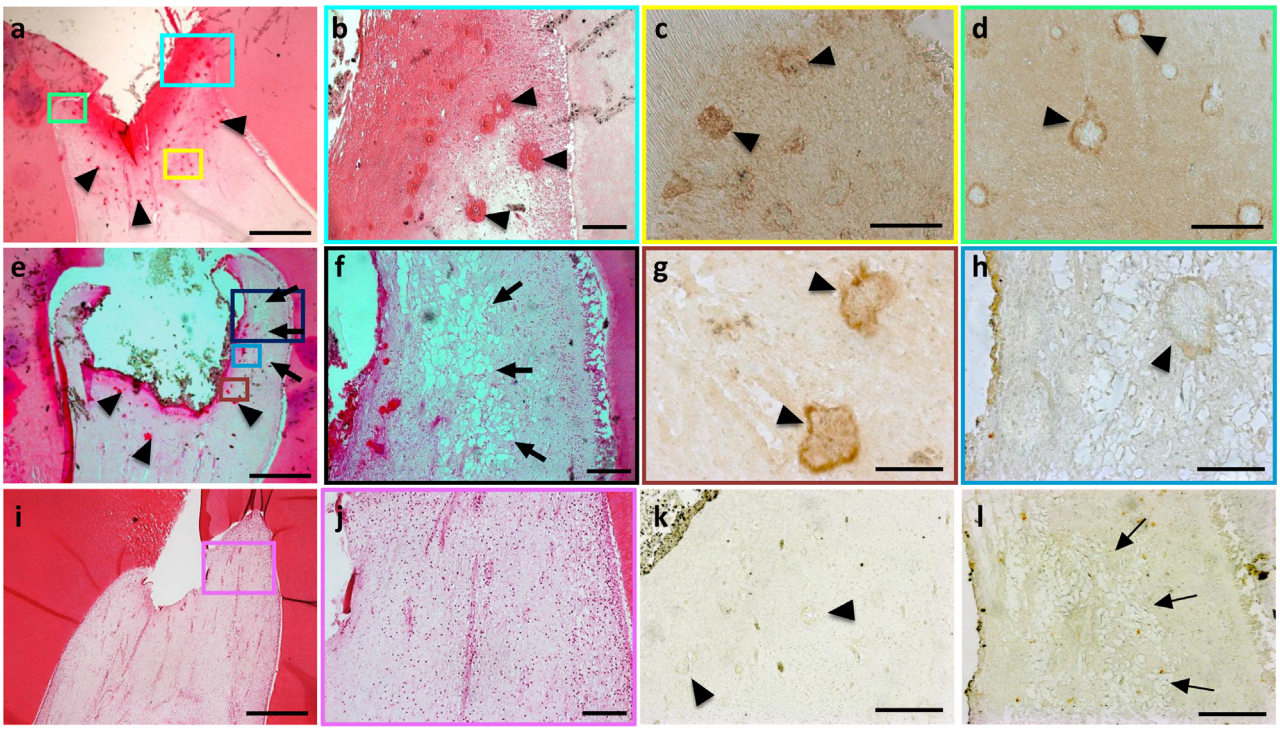

**Figure 3.** The effect of capping biomaterials on IL-8 secretion. (*A*) The secretion of IL-8 by pulp fibroblasts was significantly induced by extracts of TheraCal at 24 and 48 hours and to a lesser extent with Biodentine at 48 hours ($P < .05$, $n = 3$). The results are expressed in pg/mL as means ± standard deviation. (*B*) In entire tooth cultures, IL-8 was secreted in the cultured media of all teeth after 24 hours. However, after 48 hours, a significant increase of IL-8 release was observed from teeth with cavities filled with TheraCal compared with that of Biodentine or MEM control medium ($P < .05$, $n = 3$). The results are expressed as mean IL-8 concentration in pg/mL/apex area in $cm^2$ ± standard deviation. *A significant difference compared with the control. **Significant differences between Biodentine and TheraCal conditions ($P < .05$).

monomers (19). If unpolymerized, the free monomers can diffuse through dentin and reach the underlying pulp where they can exert their toxic effects (20–22).

In this work, we have also checked the effect of both biomaterials on DSP and nestin expression in DPSCs. Although this was a qualitative study, Biodentine induced a higher expression of the 2 markers. The absence of Biodentine toxicity and its capacity to induce the expression of DSP and nestin are in agreement with previously published data investigating Biodentine interactions with pulp cells. Indeed, it has been reported that Biodentine is biocompatible and induces odontoblastic differentiation and mineralization in cultured pulp cells (15–17). In order to investigate the impact of these biomaterials on the inflammatory response, we investigated the role of IL-8 as the key proinflammatory cytokine involved in the inflammatory process. The significant increase of IL-8 observed after incubating the cells with TheraCal-conditioned media for 24 or 48 hours suggests an amplification of the inflammatory reaction, which might compromise the pulp regenerative capacity. This result has been confirmed by entire teeth culture results, which showed a significant increase in IL-8 released in the culture media from teeth with cavities filled with TheraCal. Although a single proinflammatory cytokine was measured, its increase under TheraCal in both cell and entire tooth cultures confirmed the observed toxicity with this material. The entire tooth culture model showed that the teeth with cavities filled with Biodentine had a high or moderate number of mineralization foci with normal pulp morphology. These results are in agreement with studies *in vivo*. Indeed, when Biodentine was applied directly onto the dental pulp of rat teeth, dentin bridge formation was obtained after 14 days (23) and after pulpotomy in pig teeth (24). Similarly, a dentin bridge was obtained 6 weeks after capping with Biodentine in human teeth (2). This induction of mineralization seems to be caused by transforming growth factor beta 1 release from pulp fibroblasts (25). This factor is involved in the chemotaxis of pulp stem cells to the material application site and their differentiation into odontoblastlike cells (26). By contrast, the pulps capped with TheraCal were partly or completely disorganized, and a moderate number of foci (6/11) or none (5/11) was observed. Data on TheraCal direct capping in human teeth or in laboratory animals are lacking. However, it has to be pointed out that, under normal conditions, the photopolymerization of resinous materials is never complete, and it is reported that only 50%–75% of the double bonds can react on setting (27, 28). This explains why when adhesive resins were tested in direct pulp capping applications, they were unable to induce reparative dentin, suggesting that they are not recommended in vital pulp therapy (29, 30). Similarly, our previous works have shown that adding resin monomers in the culture medium inhibited DSP secretion by pulp cells (31). This has been confirmed in dog teeth in which a decreased mineralization and an incomplete dentin bridge formation had been observed after 4 weeks of TheraCal application (10). TheraCal is presented in a ready-to-use syringe to be used for direct placement in deep cavities and for direct pulp capping. The material sets by light curing for 20 s/mm increment. On the other hand, Biodentine is developed as a permanent dentin substitute that can be used as a bulk replacement material for the missing dentin in the crown and the root. It is presented as liquid and powder to be mixed with an amalgamator, and the material sets after 12 minutes. When Biodentine is used in pulp capping procedures, it penetrates into the dentin tubules forming taglike structures that provide a marginal sealing preventing bacterial infiltration (32). Thanks to its excellent mechanical properties, Biodentine can also be used as a temporary enamel substitute for up to 6 months (33). Thus, both Biodentine and TheraCal have the required mechanical properties for an overlying restoration under function (34). Additionally, tricalcium silicate materials are known for releasing calcium and hydroxide ions, which induce the mineralization processes when used in a direct contact with the pulp (35). Thus, the idea of incorporating resins to reduce the setting time of tricalcium silicate–based materials to less than 1 minute appeared seducing. However, the placement of resins directly onto the pulp may compromise its vitality. Indeed, experimental works on TheraCal showed its toxicity to pulp cells *in vitro* (9) and *in vivo* (10), whereas data in human teeth are still lacking. Also, it remains unclear why releasing dental restorative materials does not undergo rigorous evaluation with all steps from *in vitro* to clinical trials before their release.

Although this is a short-term study *in vitro*, the entire culture model used here has been validated previously for studying the early steps of dentin regeneration and for studying the effects of pulp capping materials on pulp mineralization (18, 25). Thus, it appears more adapted to study capping materials' toxicity to the pulp than isolated cell cultures (9) and clearly shows that adding resins to tricalcium silicates alters their bioactive potential and leads to pulp toxicity. This highlights that choosing the appropriate biomaterial is of utter

A




B

| | | Control (n=11) | TheraCal® (n=11) | Biodentine™ (n=11) |
|---|---|---|---|---|
| Mineralization potential | High | 0[a] | 0[a] | 6[a] |
| | Moderate | 0[a] | 6[a] | 2[a] |
| | None | 11[a] | 5[a] | 3[a] |
| Pulp tissue organization | Normal | 8[b] | 3[b] | 6[b] |
| | Disorganized beneath the cavity | 3 | 3 | 4 |
| | Complete pulp disorganization | 0[b] | 5[b] | 1[b] |

**Figure 4.** Direct pulp capping with Biodentine and TheraCal using a human tooth culture model. (*A*) The histologic results and immunolabeling of the pulp capped with (*a*, *b*, *c*, *d*, and *k*) Biodentine, (*e*, *f*, *g*, *h*, and *l*) TheraCal, and (*i* and *j*) control cavities. (*a* and *b*) Numerous foci of various sizes (*arrowhead*) are visible in the pulp area located beneath Biodentine. (*e* and *f*) A large disorganization area (*arrow*) can be observed in the pulp beneath TheraCal with scattered mineralized foci (*arrowhead*). (*i* and *j*) A normal pulp was observed under control cavities without mineralization. Nestin was expressed in mineralized foci and sequestered cells beneath cavities filled with (*e*) Biodentine and (*f*) TheraCal. DSP was strongly expressed at the periphery of the mineralized matrix foci under (*g*) Biodentine but showed a weak expression under (*h*) TheraCal. (*k* and *l*) No DSP or nestin staining was detected in the negative controls when isotypes were used. Scale bars represent 500 $\mu$m in *a*, *e*, and *i* and 100 $\mu$m in *b*, *c*, *d*, *f*, *g*, *h*, *j*, *k*, and *l*. (*B*) Pulp response evaluation to direct pulp capping materials compared with the control. Subgroups with different superscript letters were statistically different ($P < .05$).

importance for a favorable vital pulp therapy outcome. Within the limits of this preclinical work, our data do not bring support to TheraCal for direct pulp capping.

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

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