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Induction of specific cell responses to a Ca_3SiO_5 -based posterior restorative material

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A B S T R A C T

Objectives. A Ca_3SiO_5 -based cement has been developed to circumvent the shortcomings of traditional filling materials. The purpose of this work was to evaluate its genotoxicity, cytotoxicity and effects on the target cells' specific functions.

Methods. Ames' test was applied on four *Salmonella typhimurium* strains. The micronuclei test was studied on human lymphocytes. The cytotoxicity (MTT test), the Comet assay and the effects on the specific functions by immunohistochemistry were performed on human pulp fibroblasts.

Results. Ames' test did not show any evidence of mutagenicity. The incidence of lymphocytes with micronuclei and the percentage of tail DNA in the Comet assay were similar to the negative control. The percentage of cell mortality with the new cement as performed with the MTT test was similar to that of biocompatible materials such as mineral trioxide aggregate (MTA) and was less than that obtained with Dycal. The new material does not affect the target cells' specific functions such as mineralization, as well as expression of collagen I, dentin sialoprotein and Nestin.

Significance. The new cement is biocompatible and does not affect the specific functions of target cells. It can be used safely in the clinic as a single bulk restorative material without any conditioning treatment. It can be used as a potential alternative to traditionally used posterior restorative materials.

1. Introduction

Commonly used direct restorative materials for Class I and II cavities are resin composites and amalgams [1,2]. In the early 1980s, amalgam restorations were reported to release mercury vapors which may be harmful to the environment, the dentist as well as the patient [3].

Direct composite restorations have gradually been used to replace amalgam for anterior restorations and small- to

moderate-sized posterior restorations. In contrast to amalgam, resin composites enable micro-mechanical retention by the use of different bonding techniques. Yet there is still some concern with composite resin wear resistance in high-stress situations, polymerization shrinkage, microleakage, and unreacted monomer and toxic ingredient release [4–6].

Search for a replacement for amalgam and resin composites has been ongoing for many years. Calcium hydroxide

(Dycal[®]) is one of the most widely used pulp capping agents. Its basic pH is the main reason for its apparent toxicity in vitro [7]. However, it has been demonstrated that a dentin bridge formation can be obtained with this material 3 months after capping human teeth with mild to moderate chronic inflammation, mild hyperemia and necrosis [7,8].

Recent research focused on the use of biocompatible materials such as Portland cement. Mineral trioxide aggregate developed in the 1990s as a root-end filling material has a similar constitution to Portland cement and is composed primarily of tricalcium and dicalcium silicate [9]. It is known as a biocompatible material. In vitro, a high rate of cell viability was reported with MTA extracts with a methyltetrazolium (MTT) assay [10–12]. Additionally, MTA used for pulp capping or partial pulpotomy stimulates reparative dentin and complete bridge formation in vivo after 2 months with no signs of inflammation [8,13,14]. However, the setting time of MTA is 2 h 45 min which is too long for a material to be used as a dental restorative material [15]. Moreover, the mechanical properties of both Dycal and MTA are not compatible for use as dental restorative material.

Tricalcium silicate is the main constituent of MTA, and the main raw material in Portland cement. It is known that Ca_3SiO_5 possesses hydraulic property and the spontaneous development of strength on hydration. But its setting time is too long and its compressive strength hardly reaches 20.2 MPa after 28 days to meet the need of clinical applications as a restorative material [16]. Calcium chloride is one of the most effective accelerators of hydration and setting in Portland cement pastes. Although the addition of CaCl_2 up to 15% in the liquid phase into Ca_3SiO_5 decreased the final setting time from 180 to 90 min, the compressive strength remained weak (23.46 MPa) at 7 days [17]. The use of superplasticisers as very effective dispersing agents to reduce the water content was used in fast setting Portland cements. This has been shown to lower the setting time to 7 min but the compressive resistance did not exceed 50 MPa even after 28 days [18].

Based on Portland cement properties, a Ca_3SiO_5 -based material for direct restorative posterior fillings has been developed in the authors' laboratory. The material is inorganic and non-metallic. It is composed of Ca_3SiO_5 , CaCO_3 , ZrO_2 , water and a superplasticising admixture to reduce the water content of the mix and to retain its workability. This material is presented in the form of a powder and a liquid and can be prepared by mixing with an amalgamator. The new Ca_3SiO_5 cement is compatible with working in the clinic. It has a setting time of 10 min and was developed to be used in direct and indirect pulp capping procedures as a single application bulk restorative material without any cavity conditioning treatment. Since it may be directly applied to the dental pulp, its biological properties were compared to biomaterials usually used in pulp capping procedures such as MTA and Dycal.

Since this material belongs to a new class of restorative materials, its biocompatibility is questioned and in this paper its cytotoxicity and genotoxicity are investigated. The effect it may have on the specific functions of target cells was also evaluated.

2. Materials and methods

2.1. Reagents

All materials used for culture media preparation were purchased from Gibco BRL (Life Technologies Inc., Grand Island, NY, USA) unless otherwise specified. Minimum Essential Medium (MEM) was supplemented with 10% fetal bovine serum; 100 UI/ml penicillin; 100 $\mu\text{g}/\text{ml}$ streptomycin (Biowhittaker, Gagny, France) and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B (Fungizone[®]). Chemicals were obtained from Sigma-Aldrich (Sigma Chemicals Corp., St. Louis, MO) unless otherwise stated.

2.2. Teeth

For pulp cell cultures, normal immature third molars freshly extracted for orthodontic reasons from 16 to 18 year-old patients were used after obtaining theirs and their parents' informed consent and was conducted with local ethical committee approval. Additionally, for the preparation of dentin slices, 30 healthy human third molars freshly extracted were stored at 4 °C in saline solution and used within 2 h of collection.

S. typhimurium strains TA97a, TA98, TA100, and TA102 were kindly provided by Dr. B.N. Ames (Berkeley, CA, USA).

2.3. Antibodies

Polyclonal antibodies against the type I collagen were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). Anti-dentin sialoprotein antibodies were obtained from WT Butler (UTHSC, Houston, TX, USA). Preparation and characterization of the polyclonal antibodies against dentin sialoprotein (DSP) have been already described [10]. Anti-nestin antibodies were purchased from Chemicon International (Temecula, CA, USA).

This work was performed on a new Ca_3SiO_5 -based cement developed with an industrial partner (Laboratoires Septodont, Saint Maur des Fosses, France). MTA (Dentsply Tulsa dental, Tulsa, OK, USA) (batch number 0203332604) and Dycal (De Trey Dentsply, Milford, DE, USA) (batch number 0204000983) were used as a reference material for cytotoxicity tests.

2.4. Toxicity by indirect contact between the biomaterial and the culture media

2.4.1. Preparation of the dentin slices

From the third molars, thirty dentin slices were prepared with a low speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) with water coolant. The dentin sections were from areas adjacent to the pulp chamber, but they showed no evidence of inclusion of a pulpal horn. The dentin slices had a thickness of 0.7 ± 0.05 mm. To create a constant dentin surface area, a Plexiglas ring 1 cm thick, 2 cm in diameter with a hole of 0.8 cm in its center was placed on the pulpal side of the dentin slice and was attached with a non-cytotoxic cyanoacrylate glue. This permitted us to reduce and to standardise the

exposed dentin surface area to 50.24 mm². The coronal side of the dentin slice was covered with 1 mm thick MTA (n = 10), new cement (n = 10) and Dycal (n = 10). The reference materials were applied according to the manufacturers' instructions. The new Ca₃SiO₅ cement was prepared by mixing the recommended quantities of liquid and powder and vibrating with an amalgamator. It was applied without any conditioning treatment.

2.4.2. Simulation of pulpal pressure

The Plexiglas rings and the dentin slices were placed in a special device used to simulate a pulsatile pulpal pressure, as previously described [19]. The Plexiglas device was used to maintain the dentin slice in such a position that the MEM culture medium slightly touched the pulpal side of the dentin slice while the coronal side was open to the atmosphere. The lower chamber (4 ml), in contact with the pulpal side of dentin contained the culture medium. A pulsatile pulpal pressure (12–18 cm H₂O) was applied. The dentin slices were inserted in the Plexiglas device for 24 h. After 24 h, the media were collected and called the indirect contact media.

2.5. Toxicity by direct contact between the biomaterial and the culture media

Ten samples of each material were prepared according to manufacturer recommendations and stored in an incubator prior to sterilization with UV rays. The Ca₃SiO₅ cement was prepared as described above. The samples were stored in 1 ml MEM with 10% foetal calf serum supplemented with penicillin 100 IU/ml and streptomycin 100 µg/ml for 24 h. According to ISO standards, the ratio between the surface of the sample and the volume of medium was 0.5 cm²/ml. The resulting pH values in the buffered culture medium were: Dycal 9.2; MTA 8.1 the Ca₃SiO₅ cement 8.2. These media were called the direct contact media (n = 10 per material).

2.6. MTT assay

Pulpal fibroblasts were plated at 30,000 cells cm⁻² in 96-well plates (Falcon 3072, Becton Dickinson, Oxford, GB). The 96-well dishes were then placed into a humid incubator with an atmosphere of 5% CO₂, 95% air for 24 h prior to use. After this 24 h period, the medium from the 96-well plates was removed and replaced by the test medium. At that time, the 96-well plates were placed in an incubator again for 24 h. A succinyl dehydrogenase assay (MTT) was performed on the dishes after 24 h of incubation (i.e., 48 h after the beginning of the experiment). The medium was removed and immediately replaced with 100 µl/well of a 0.5% of 3-(4,5-dimethylthiazol-2-yl)-2,(-diphenyl tetrazolium bromide) in the medium. After incubation for 2 h at 37 °C, the supernatant was discarded, and the formazan crystals were solubilized with 100 µl/well of dimethyl sulfoxide (DMSO). The absorbance of each 96-well dish was measured using an automatic microplate spectrophotometer (E 960, Bioblock, Strasbourg, France) at 550 nm.

For direct and indirect contact media, a two-way analysis of variance (medium dilution and material), followed by a Dun-

can test, was used to compare the cytotoxicity of MTA, the new Ca₃SiO₅ cement and Dycal.

In order to study the long term effects on the pulp fibroblasts differentiation, it is known that lower concentrations can be toxic after long term incubation with cells. Thus, the medium used for the next part of the study was one which decreased the MTT activity by less than 5%.

2.7. Influence of the new Ca₃SiO₅ cement and MTA on the differentiation of pulp fibroblasts

In order to evaluate the effect of the new Ca₃SiO₅ cement and MTA on the differentiation of pulp fibroblasts, the cultured cells were incubated in the conditioned MEM medium obtained after direct and indirect contact with the materials supplemented with 2 mM β-glycerophosphate. The cells were cultured for 4 weeks in cell culture chambers and the media were changed every other day. After culture, the cells were fixed with 70% ethanol for one hour at 4 °C and processed for immunohistochemistry. The effect of the materials on the cytodifferentiation was evaluated by studying the specific protein expression of control cells compared to that of cells cultured with the medium after being in contact with the test material [20].

2.7.1. Immunohistochemistry

The cells were permeabilized for 15 min with 0.5% Triton X-100 in PBS. Primary antibodies were diluted in PBS containing 0.1% Bovine Serum Albumin (BSA). The incubation with primary antibodies was performed overnight at 4 °C. Anti-collagen I antibodies were used at 40 µg/ml and anti-nestin antibody at 5 µg/ml. Anti-dentin sialoprotein antibody was diluted 1:200 in PBS. Immunostaining was revealed using the labeled streptavidin-biotin kit (LSAB; Dako Corporation, Carpinteria, CA, USA) according to the manufacturer's instructions. Glycer-gel was used as a mounting medium (Dako Corporation). Controls were performed by omitting primary antibodies or incubation with unrelated primary antibodies (cytokeratin 19). All controls were negative.

2.8. Genotoxicity assays

2.8.1. Ames test

S. typhimurium TA97a, TA98, TA100, and TA102 strains were grown overnight from frozen cultures in Oxoid nutrient broth No. 2 for 10–12 h. Mutagenicity assays were performed as described [21]. The genotype of each *S. typhimurium* tester strain was confirmed in each experiment, and negative and positive controls were routinely included.

After the preparation and setting of the cement, it was ground to prepare a stock solution prior to testing by adding 60 mg of the cement in 1 ml of Nutrient Broth No. 2 (NB 2) medium or DMSO solvent for 24 h at 37 °C under mixing. These stock solutions from two independent experiments were then tested in triplicate and results from both experiments in NB 2 and DMSO are presented. Increasing volumes of test samples (4, 6, 8 and 10 µl) were incubated with each bacterial strain for 60 min at 37 °C under mixing. The mixture consisting of bacteria and a test compound was plated on plates in VB medium. The bacteria were then incubated at 37 °C

for 48 h and revertant colonies were counted with an automated colony counter (Spiral System Instruments, Bethesda, MS, USA). The experiments were carried out in the presence and in the absence of an S9 fraction isolated from liver of phenobarbital/ β -naphthoflavone-treated rats. This S9 fraction (4%) was routinely included in an S9-Mix, and the amount of protein was adjusted to 1.25 mg protein per plate. A substance was qualified positive if it induced a dose-related and reproducible increase in the numbers of revertants or twice as many spontaneous revertants per plate [22].

2.9. Micronucleus test

This work was performed on lymphocytes obtained by vein puncture from 6 healthy non-smoking donors, after informed consent, and collected in glass tubes containing lithium heparin anticoagulant according to Digue et al. [23]. Briefly, cultures were carried out by adding 0.7 ml of whole blood to 9.3 ml of X-VIVOTM Medium (Bio-Whittaker, Belgium) supplemented with 25% fetal calf serum (Gibco, Life TechnologiesTM, Germany), heparin (50 U/ml), and antibiotics (penicillin 100 U/ml and streptomycin 100 μ g/ml). The cells were stimulated with phytohemagglutinin (1 mg/ml), a specific mitogen agent of human T-lymphocytes. The cells were then cultured for 72 h at 37 °C in a humidified atmosphere containing 5% CO₂.

The Ca₃SiO₅ cement extract was prepared as described above in the culture medium or DMSO and added to the culture at 24 h. The cells were directly exposed to serial dilutions (1%, 2.3%, 3.7%, and 5%) of the cement extracts for 48 h. Negative control was achieved by adding DMSO at a final concentration of 0.1%. Mitomycin C, used as a reference genotoxic agent, was used as positive control 5 μ g/ml. Cytochalasin B was added to the culture (5 μ g/ml) 44 h after PHA stimulation.

The cultures were stopped at 72 h and the cells harvested by centrifuging (10 min at 360 g). They were then treated by a mild hypotonic treatment (1 min in KCl 0.075 M) and immediately fixed with methanol:acetic acid (3:1). This fixing step was repeated twice after 20-min storage at 4 °C. Cells were smeared on pre-cleaned microscope slides and air-dried. Staining was performed with 5% Giemsa in Milli-Q water for 15 min.

Stained slides were coded and scored by light microscopy at 400 \times magnification. For each slide, 1000 Giemsa-stained binucleated lymphocytes with a well-preserved cytoplasm were scored for the presence of micronuclei. In the micronucleated binucleated cells, the number of MN per cell was recorded. Micronuclei were expressed in terms of micronucleated cells per 1000 binucleated lymphocytes. All the slides were examined twice by the same scorer. As a measure for toxicity, the binuclearity index (BI) was determined by scoring the binucleated cells for 1000 lymphocytes (mono- and binucleated cells) and linked to the percentage of lymphocytes that produced complete cell division for the different drugs tested, and then provided an index of cytotoxicity [24]. An extract of a material was considered positive if at least a three-fold increase of the numbers of micronuclei over negative controls was observed at one or more dilutions of the original extract [25,26].

2.10. Single-cell gel (Comet) assay

The Ca₃SiO₅ cement extract was prepared and put in MEM medium (60 mg/ml) for 24 h at 37 °C under mixing. The cells were directly exposed to serial dilutions of the cement extracts for 2 h. The protocol used for single-cell gel (Comet) assay followed the guidelines proposed by Tice et al. [27]. Briefly a volume of 10 μ l of cells (10⁴ cells) of each treatment was added to 120 μ l of 0.5% low-melting-point agarose at 37 °C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris-HCl buffer pH 10, 1% sodium sarcosinate with 1% Triton X-100, and 10% DMSO) for about 1 h. Prior to electrophoresis, the slides were left in alkaline buffer (pH >13) for 20 min and electrophoresed for another 20 min, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 mol/l Tris-HCl (pH 7.5) fixed in absolute ethanol, and stored at room temperature until analysis blindly in a fluorescence microscope at 400 \times magnifications. In order to minimize extraneous DNA damage from ambient ultraviolet radiation, all steps were performed with reduced illumination. An automatic analysis system (Comet Assay II; Perceptive Instruments, Haverhill, UK) was used to determine DNA damage. Tail moment (product of tail DNA/total DNA by the center of gravity) was considered to estimate DNA damage from 50 cells per treatment.

3. Results

3.1. Determination of the toxicity with or without dentin disc interposition

When the toxicity was evaluated indirectly through a dentin slice, the analysis of variance failed to show a statistical difference between the new cement, Pro Root MTA, and Dycal (ns) (Table 1). None of the materials was cytotoxic. However, when the toxicity was evaluated without dentin slice interposition, the analysis of variance showed a statistically significant difference among the three materials ($P < 0.001$). The Duncan test

Table 1 – Cytotoxicity after indirect contact between the materials and culture medium through a dentin disc

| | New Ca ₃ SiO ₅ cement | MTA | Dycal |
|-----------|---|------------|------------|
| Undiluted | 0 \pm 8% | 0 \pm 9% | 0 \pm 8% |
| 50% | 0 \pm 4% | 0 \pm 4% | 0 \pm 4% |
| 10% | 0 \pm 4% | 0 \pm 3% | 0 \pm 4% |

The new Ca₃SiO₅ cement, MTA, and Dycal were applied on the coronal side of the dentin slices in Plexiglass devices with pulp pressure simulation. After 24 h, the culture media in contact with the pulpal side of the dentin slices were used to determine cell viability. The pulp fibroblasts were incubated with these media (either undiluted, or diluted in the culture medium to 50% or to 10%) for 24 h before applying the MTT test on human pulpal fibroblasts. Optical density values of untreated control cultures normalized to 100% was in the range of 0.9–0.95. The results are expressed as mean cell toxicity \pm S.D.

Table 2 – Cytotoxicity after direct contact between the material and culture medium

| | New Ca ₃ SiO ₅ cement | MTA | Dycal |
|-----------|---|--------|----------|
| Undiluted | 0 ± 8% | 0 ± 9% | 22 ± 10% |
| 50% | 0 ± 5% | 0 ± 5% | 10% ± 5% |
| 10% | 0 ± 4% | 0 ± 3% | 2 ± 2% |

The cytotoxicity of the new cement compared to MTA and Dycal on human pulp fibroblasts was evaluated after 24 h contact between the materials and the culture medium (either undiluted, or diluted in the culture medium to 50% or to 10%) with the MTT test. Both were less cytotoxic than Dycal ($P < 0.001$). The results are expressed as mean cell toxicity ± S.D.

showed that Dycal displayed a higher cytotoxicity than MTA and the new Ca₃SiO₅ cement (Table 2).

According to this study, a dilution of 10% was chosen for studying the materials' effects on fibroblasts specific functions because it has biological effects without being toxic.

3.2. Influence of the two materials on pulp fibroblasts differentiation into odontoblastic cells

Control cells expressed collagen I, dentin sialoprotein and Nestin. Pulp fibroblasts secreted a mineralized matrix and the cells, particularly those contacting the mineralized matrix, expressed Nestin (Figs. 1 and 2).

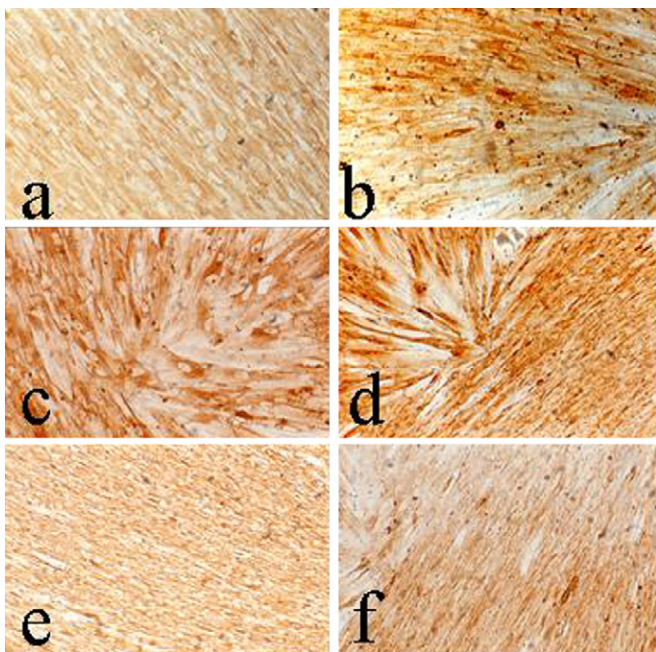


Fig. 1 – Effect of the new Ca₃SiO₅ cement on pulp fibroblast specific gene expression. Immunohistochemistry was used to evaluate the effect of the new Ca₃SiO₅ cement and MTA on pulp cells specific genes expression. Control cultures express collagen type I (a) and dentin sialoprotein (b). When the media containing the new Ca₃SiO₅ cement (c and d) and MTA (e and f) extracts were added to the cultures for 4 weeks, collagen I (c and e) and dentin sialoprotein (d and f) were also expressed at a high level in the pulp cells. Original magnifications = × 10.

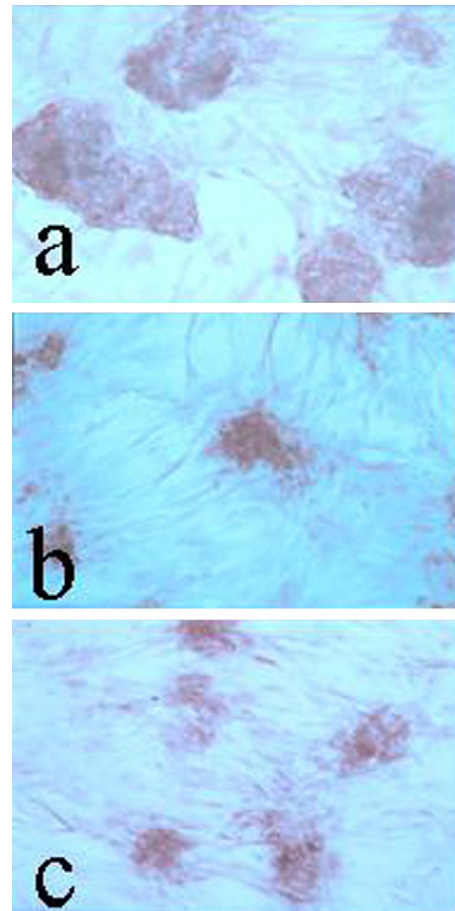


Fig. 2 – Effect of the new Ca₃SiO₅ cement on pulp cells mineralization. Immunohistochemistry was used to evaluate the effect of the new Ca₃SiO₅ cement and MTA on pulp cells differentiation and mineralization. Control cultures express Nestin and secrete a mineralized matrix in the form of nodules (a). When the media containing the new cement (b) or MTA (c) extracts were added to the cultures for 4 weeks, a mineralized matrix deposition was also observed. Nestin was also expressed at a high level in pulp cells and its expression was stronger in the mineral nodules forming cells. Original magnifications = × 10.

After adding the media containing extracts of the new Ca₃SiO₅ cement or MTA to the cultured pulp cells, collagen I, dentin sialoprotein were strongly expressed by the pulp cells (Fig. 1). Mineral nodule formation was also observed (Fig. 2). Nestin was expressed by the cells but not in the mineral nodules. The immunostaining intensity was always higher in cells forming the mineral nodules than the cells away from these nodules.

3.3. Genotoxicity

Ames' test did not show any evidence of mutagenicity of the Nutrient Broth No 2 medium after being in contact with the new cement, whatever the dilution of the test medium (Table 3). The mutations observed with the new cement were comparable to the spontaneous reverse mutations obtained in

Table 3 – Mutation frequencies of Ames tester strains using the liquid preincubation assay

| Metabolic activation (S9 mix ^a) | Product | Volume (μl) | Number of revertants/plate (mean ± S.D.) | | | |
|--|--|-------------|--|----------|-----------|-----------|
| | | | TA 97a | TA 98 | TA 100 | TA 102 |
| + | NB No. 2 | 10 | 171 ± 9 | 24 ± 3 | 136 ± 4 | 382 ± 17 |
| + | DMSO | 10 | 166 ± 7 | 25 ± 1 | 125 ± 13 | 355 ± 16 |
| – | NB No. 2 | 5 | 183 ± 13 | 26 ± 5 | 135 ± 11 | 402 ± 18 |
| – | DMSO | 5 | 191 ± 11 | 27 ± 4 | 138 ± 9 | 423 ± 26 |
| + | New Ca ₃ SiO ₅ cement (NB No. 2 extract) | 4 | 162 ± 14 | 30 ± 1 | 135 ± 14 | 360 ± 10 |
| | | 6 | 177 ± 5 | 26 ± 1 | 120 ± 2 | 397 ± 15 |
| | | 8 | 177 ± 4 | 29 ± 5 | 132 ± 5 | 351 ± 7 |
| | | 10 | 192 ± 4 | 27 ± 4 | 150 ± 13 | 345 ± 2 |
| – | New Ca ₃ SiO ₅ cement (NB No. 2 extract) | 2 | 215 ± 11 | 25 ± 1 | 161 ± 10 | 500 ± 24 |
| | | 3 | 223 ± 9 | 25 ± 3 | 172 ± 21 | 424 ± 36 |
| | | 4 | 225 ± 15 | 25 ± 1 | 160 ± 35 | 439 ± 3 |
| | | 5 | 205 ± 23 | 23 ± 1 | 182 ± 12 | 517 ± 44 |
| + | New Ca ₃ SiO ₅ cement (DMSO extract) | 4 | 170 ± 19 | 29 ± 2 | 119 ± 3 | 334 ± 49 |
| | | 6 | 189 ± 3 | 25 ± 2 | 126 ± 13 | 376 ± 3 |
| | | 8 | 175 ± 2 | 28 ± 8 | 145 ± 1 | 336 ± 24 |
| | | 10 | 164 ± 23 | 43 ± 7 | 136 ± 5 | 314 ± 11 |
| – | New Ca ₃ SiO ₅ cement (DMSO extract) | 2 | 193 ± 2 | 35 ± 2 | 149 ± 3 | 421 ± 5 |
| | | 3 | 186 ± 5 | 37 ± 5 | 117 ± 8 | 445 ± 42 |
| | | 4 | 224 ± 17 | 27 ± 3 | 140 ± 6 | 463 ± 26 |
| | | 5 | 173 ± 8 | 30 ± 1 | 144 ± 3 | 435 ± 36 |
| + | B[a]P | 0.5 μg | 1121 ± 37 | 423 ± 26 | 1000 ± 87 | 679 ± 28 |
| – | ICR 191 | 0.02 μg | 553 ± 21 | NT | NT | NT |
| – | 2,4,7-TNFone | 0.02 μg | NT | 165 ± 3 | NT | NT |
| – | NaN ₃ | 0.5 μg | NT | NT | 585 ± 12 | NT |
| – | MitC | 0.2 μg | NT | NT | NT | 3658 ± 54 |

After preparation and setting of the cement, it was grinded prior to testing. 60 mg of the cement were placed in 1 ml of Nutrient Broth No 2 or DMSO solvent for 24 h at 37 °C under mixing. The stock solutions from two independent experiments were tested in triplicate, and results from both experiments in NB 2 and DMSO are presented. Increasing volumes of test samples (4, 6, 8 and 10 μl) were incubated with the each of the bacterial strains for 60 min at 37 °C under mixing. The mixture consisting of bacteria and a test compound was plated on plates in VB medium at 37 C for 48 h and revertant colonies were counted. The experiments were carried out in the presence and in the absence of an S9 fraction. The test was qualified positive if it induced a dose-related and a reproducible increase of the numbers of revertants or twice higher than the spontaneous revertants per plate. All data are expressed as means ± S.D. Positive controls were Benzo[a]pyrene (0.5 μg) with S9 MIX for all strains. Positive controls were 2-methoxy-6-chloro-9-(3-(2-chloro-ethyl)aminopropylamino)acridine (ICR 191, 0.1 μg) for TA97a; 2,4,7-trinitro-9-fluorenone (2,4,7-TNFone, 0.02 μg) for TA98; sodium azide (NaN₃, 1 μg) for TA100 and mitomycin C (MitC, 0.05 μg) for TA102 without S9 MIX. NT: non-tested.

^a The S9 MIX included 4% S9, 4.2 mM NADP and 5.2 mM G6P.

the controls performed with the NB 2 and DMSO solvent. The results show that the new Ca₃SiO₅ cement does not induce reverse mutations either with or without the S9 metabolic activation system. Similar results were obtained with all bacterial strains tested.

Table 4 – Micronucleated human lymphocytes count in Ca₃SiO₅ cement-treated cultures

| Ca ₃ SiO ₅ cement dilution | Micronucleated lymphocytes (%) ± S.D. |
|--|---------------------------------------|
| 1% | 4.0 ± 1.1 |
| 2.3% | 4.0 ± 1.1 |
| 3.7% | 4.0 ± 1.2 |
| 5% | 4.2 ± 1.2 |
| Negative control ^a | 3.7 ± 1.2 |
| Positive control ^b | 16.0 ± 6.0*** |

Comparison with the control: ***P < 0.001.

^a Culture medium X-VIVO 10.

^b Mitomycin C 5 μg/ml.

The micronuclei test revealed that after incubating the lymphocytes with different dilutions of the new cement, the rate of lymphocytes with micronuclei was similar to that obtained with the negative control. It ranged from 3.9% to 4.1% with increasing concentrations (1–5%) in aqueous or hydrophobic medium. The positive control showed a rate of 16% (Table 4).

The Comet assay performed with serial dilutions of the new Ca₃SiO₅ cement on human pulp fibroblasts revealed that the percentage of DNA in the tail ranged from 12.59 for the 0.1% dilution to 15.58 with undiluted medium. This percentage was 13.19 with the negative control and 46.52 with the positive control (Table 5).

4. Discussion

The biocompatibility of the new cement is shown in this study by the absence of cytotoxicity and genotoxicity and the fact that the new material does not affect the cytodifferentiation of human pulp fibroblasts in odontoblastic cells.

| Ca ₃ SiO ₅ cement dilution | Tail DNA (%) mean ± S.D. |
|--|--------------------------|
| 0.1% | 12.59 ± 0.96 |
| 1% | 13.31 ± 0.88 |
| 10% | 14.90 ± 1.06 |
| Undiluted | 15.58 ± 1.08 |
| Negative control ^a | 13.19 ± 0.96 |
| Positive control ^b | 46.52 ± 1.45*** |

Comparison with the control: ***P < 0.001. NS: non-significant.

^a 0.1% DMSO.

^b H₂O₂ (13.2 mM).

Although Portland cements are known as non-toxic, in this work, 3 tests were performed to evaluate the genotoxicity of the new Ca₃SiO₅ cement after solubilisation in hydrophilic or hydrophobic conditions. These tests were performed because the cement developed here contains a modified polycarboxylate in the superplasticizer. It has been reported that polycarboxylate (Aqualox[®]) elicited mutagenic effects on *S. typhimurium* TA 98 and TA 1535. In the presence of S9 fraction, Aqualox[®] elicited weak mutagenic effects on *S. typhimurium* TA 1535 and dose-dependent mutagenic effects on *S. typhimurium* TA 98 [28]. Here, Ames' test performed with and without the S9 fraction on 4 different bacterial strains including TA 98 failed to detect significant reverse mutations.

While Ames' test was performed on prokaryotic cells, the micronucleus test and the Comet assay were performed on eukaryotic cells. The micronucleus test was important to perform in order to detect any structural chromosomal alteration in the host cells involved in the defense mechanisms. It revealed that no chromosomal damage was found with the material. The Comet assay was developed as reliable biochemical technique for evaluating DNA damage and breaks in single mammalian cells [27]. This test was performed on the target cells of the new cement and did not show significant DNA breaks in human pulp fibroblasts. These results may be explained either by the fact that the modification of polycarboxylate suppressed its mutagenic effects or by the fact that its concentration is too low in the cement to have any mutagenic effect.

The new material was developed as a restorative material both for direct and indirect pulp capping. That is why toxicity was investigated under two conditions: indirectly through dentin discs and directly by applying the medium containing the new cement extract on the target cells. The new cement was not toxic to the cells under either condition even when tested undiluted.

The toxicity of the new cement was compared to materials used in pulp capping situations. This study confirms the absence of MTA toxicity. This material was introduced in the 90s and is well accepted by endodontists as an excellent material for retrofilling, perforation repair and apexification. This success is due, in part, to the sealing properties of the material [15] but mainly to its biocompatibility [29,30]. It has been shown that using the same MTT assay that MTA was non-toxic to periodontal ligament fibroblasts [10] and human gingival fibroblasts [31]. The current results corroborate those of two other indirect contact studies using agarose superimposition

[32] or millipore filter [33]. This total absence of toxicity possibly explains the adhesion of human osteoblasts to the material surface [34].

Dycal was slightly cytotoxic in direct contact. This confirms previous work [7] and may be due to the solubility of salt resulting from the reaction between salicylic acid and zinc oxide releasing zinc ions and non-reacting hydroxide ions. It is possible that this is clinically irrelevant because 20% cell death without pulpal clearance does not represent harmful behavior of the material. In vivo, Dycal does not elicit an inflammatory reaction after intramuscular implantation in rats [35] and induces slight inflammation after direct pulp capping [36]. The toxicity decrease after dentin disc interposition is in agreement with previous work on the importance of dentin thickness and hydraulic conductance on restorative material toxicity [37].

All studies comparing the effects of MTA versus Dycal concluded a higher efficiency of MTA. Direct pulp capping with MTA gave better results than Dycal at 4 months on human wisdom teeth [8] and at 2 months in dog teeth [38].

Absence of toxicity with the new cement was comparable to that of MTA and this was the case either with or without dentin slice interposition. Additionally, both the new cement and MTA do not seem to affect the odontoblastic specific protein expression or mineralization.

In previous work, the authors have shown that pulp cells cultured with β-glycerophosphate secrete an extracellular matrix deposit which progressively forms nodules of mineralized material. FTIR analysis showed that it was a specific deposition which had the same mineral composition of dentin [39]. The cultured cells, particularly those involved in mineral nodule formation, express a high level of alkaline phosphatase activity indicating high mineralization potential of these cells. In addition, the cells involved in the mineralization express the type I collagen, osteonectin, DSP and Nestin. In this work, the cells treated with the new cement or MTA expressed collagen I, dentin sialoprotein and Nestin and synthesized a mineralized matrix. Collagen I is the major dentin matrix organic protein [40]. DSP which is expressed during human tooth development is a 53-kDa glycoprotein accounts for 5–8% of the dentin extracellular matrix. It is localized mainly in dental tissues and its expression was reported to be localized and confined to differentiating odontoblasts, with a transient expression in the presecretory ameloblasts [41]. However, odontoblasts express DSP to a much greater extent than other cell types [42]. Additionally, Nestin which is a human odontoblast specific intermediate filament protein [43] was expressed in these cells after adding β-glycerophosphate with a stronger expression in the cells contacting the mineral nodules.

This is of prime importance in the clinic. Coronal restorations may be placed on teeth where the odontoblastic layer is partially destroyed, making the differentiation of secondary odontoblasts necessary prior to pulp healing. The presence of toxic compounds such as monomers may interfere with this critical step of pulp healing [44].

The expression of these specific proteins by human pulp fibroblasts in the presence of MTA has never been reported, but the potential of MTA to induce cell cytodifferentiation has already been shown in animal studies. The root end closure

with MTA [45] and growth of new cementoblasts in direct contact with MTA used as a retrofilling material have been shown in dogs [46] and monkeys [47] and reparative dentin can be seen after direct pulp capping with MTA [13,38,48]. The advantage of the new material over both Dycal® and MTA resides in the fact that, in addition to its biocompatibility, its mechanical and physical properties strongly suggest its future utilisation as a bulk restorative material and not only as a pulp capping agent.

5. Conclusions

The results of the current study need to be confirmed in vivo and suggest that this new Ca₃SiO₅ cement could be used as a direct pulp capping agent but also as a lining agent. This material would possibly induce the secretion of reactionary dentin often considered as a preliminary step for pulp healing after caries removal. The good handling properties of this material associated with its biological, mechanical and physical properties let us think that this material could be used as a pulp capping agent and as a bulk restorative material at the same time. In addition, no preliminary conditioning of the cavities is required with this new cement. This would greatly simplify pulp capping techniques.

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