In vitro cytotoxicity of resin-reinforced glass ionomer cements

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Summary

Previous studies indicated that toxic components can be released from glass ionomer cements and diffuse through dentinal tubules into the pulp. This in vitro study compared the cytotoxic effects produced to pulpal fibroblasts by the leachables of the primer systems and the diffusates through dentin of four resin-reinforced glass ionomer cements (Vitremer Tri Cure System, Dyract-Prime & Bond 2.0, Dyract-Prime & Bond 2.1 and Photac-Fil). For the primers, elution samples were directly prepared in tissue culture medium. The glass ionomer cements were applied to dentin disks, after which the pulpal side of the dentin was placed in contact with 1 mL of cell-culture medium. The medium was collected at 24 h intervals through 168 h, and was placed on monolayers of human pulpal fibroblasts for 24 h. The response of the cells was assessed by succinic dehydrogenase activity (MTT method). The results showed that the primers were most cytotoxic at early intervals (24–48 h). The four resin-reinforced glass ionomer cements released sufficient components through dentin to cause suppression of cellular metabolism. Materials were most cytotoxic at early intervals and were generally less cytotoxic at later intervals, but a persistent 20% suppression of cellular metabolism was still observed with the Vitremer material at late intervals (168 h). It is concluded that most resin-reinforced glass ionomer cements may have a potential as a pulp liner because of low toxicity. Further in vivo studies are required to confirm these results.

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Introduction

Since glass ionomer cements were first introduced in the early 70s by Wilson & Kent (1972), major changes have occurred in their chemical composition. Conventional glass ionomer cements were characterized by an acid-base reaction between polycarboxilic acids and aluminosilicate glass particles that formed a set matrix. They presented favorable characteristics such as their ability to chemically adhere to dental tissues and to release fluoride ions. As reported by Mount (1995) the leaching of fluoride ions from conventional glass ionomer cements provided them with excellent anticariogenic properties. However, their clinical indications were limited because they exhibited poor mechanical and esthetic qualities (Um & Oilo 1992).

Later, lightcuring glass ionomer cements incorporating BIS-GMA and TEGDMA dental resins have been developed. With this new formulation, both the conventional acid-base reaction and the photo-polymerization of the resins produced a mixed network of glass ionomer and resin matrix. Despite improved mechanical properties and reduced sensitivity to moisture, light curing glass ionomer cements remained mostly used as liners or bases under composite resin restorations (Um & Oilo 1992). As a restorative material, the major weakness of light curing glass ionomer cements was a lack of translucency and post-operative sensitivities were frequently observed after placement (Stanley 1992).

Recently, resin-reinforced glass ionomer restorative materials have been developed to extend the clinical applications of glass ionomer cements. This new generation of material has been referred to hybrid glass ionomer cements (Friedl et al. 1995) or compomers (Krejci et al. 1994). Beside the conventional acid-base reaction and the photopolymerization of the resins, they may also develop a self-curing reaction of the resin monomers in the absence of light (Powers 1993). Recently, Carvahlo et al. (1995) reported that dentin hybridization can occur when resin-reinforced glass ionomer cements containing acidic primers are applied to dentin. Despite higher bond strength to dentin has been reported (Swift et al. 1995), there are some controversial results concerning their resistance to wear (De Gee et al. 1996). Therefore, their use as restorative material is currently limited to pediatric and geriatric dentistry (Uno et al. 1996).

Although biocompatibility studies have reported that bacterial leakage is more likely to cause adverse effects to the dental pulp than components from restorative materials (BERGENHOLTZ et al. 1982), the risk that leachables from glass ionomer cements may elicit cytotoxicity remains. When resin-modified glass ionomer cements are used, this risk is increased by several factors. To take advantage of their bonding properties, these cements must be directly applied on the tooth structure after application of acidic primers. As reported by PASHLEY (1985), acid-etched dentin will increase the diffusion of leachables relative to dentin covered by a smear layer. Second, acid etching of dentin is generally performed in an aqueous environment that interferes with the polymerization of the resins. Therefore, incompletely polymerized resin monomers are more likely to dissolve and diffuse toward the pulp (GERZINA & HUME 1994). Finally, previous *in vitro* studies have shown that either fluoride ions (LEIRSKAR & HELGELAND 1988) or resin monomers (HANKS et al. 1991) can exhibit toxicity to cultured cells. The hypothesis tested in the current study was that the addition of acidic primers and resin monomers into the composition of modern glass ionomer cements may have modified their biological properties. Specifically, the objectives of this *in vitro* study were to measure the toxic effects produced by the leachables of the primer systems and to compare the cytotoxicity of the diffusates through dentin of four resin-reinforced glass ionomer cements.

Materials and methods

Two series of experiments were performed (Fig. 1). The direct contact method determined the toxicity of the leachables from the primers and the indirect contact method determined the cellular response to the diffusates through dentin of the four glass ionomer cements (Table I). For the direct contact method, test samples of the primers were prepared by curing a thin layer of material onto a circular cover-glass for microscopy. A drop of primer was applied on the glass slide, left for 30 seconds to evaporate the solvent, gently blown with an air syringe and light cured for 40 seconds. Each sample was then placed in direct contact with 1 mL of cell culture medium and incubated at 37° C for 24 hours. After this period, the medium was collected and replaced with fresh medium for another 24 hours. The same procedure was repeated every day during the seven days of the experiment. There were eight replicates per material.

For the indirect contact method, 0.5 mm thick dentin disks were prepared at the mid-level of the crown of extracted human molars. The disks were sonicated for 30 minutes in a 3% sodium hypochlorite solution and rinsed twice with double distilled water in an ultrasonic bath. When ready for use, a polystyrene cylinder was attached to each disk by dissolving the polystyrene with chloroform. Then, each dentin disk has been controlled for permeability to fluids because great variations exist in dentin (PASHLEY 1985). Dentin permeability was measured by connecting the disk to an automated flow recording device (Flodec System De Marco Engineering Geneva CH) that automatically converted fluid flow through dentin into hydraulic conductance data (Lp: microL / min / cm^2 / cm H_2O) (CIUCCHI et al.1995). The dentin disks were apportioned into six experimental groups presenting a mean hydraulic conductance (Lp) of $1.5 \pm 0.5\ 10^{-2}$ microL/ min / cm^2/ cm H_2O. Then, the glass ionomer materials were introduced into the polystyrene tubes and applied to the disks according to manufacturer's instructions.

Table I Materials and approximate compositions

Materials	Manufacturer	Primer approximate composition*	Restorative material approximate composition*
Prime & Bond 2.0 DYRACT	De Trey-Dentsply, Konstanz, Germany	Prime & Bond 2.0: TEGDMA, Penta, Acetone	DYRACT: Fluoroaluminosilicate, Polyacrylic acids; UDMA
Prime & Bond 2.1 DYRACT	De Trey-Dentsply, Konstanz, Germany	Prime & Bond 2.1: TEGDMA, Penta, Acetone Cétylamine Hydrofluoride	DYRACT: Fluoroaluminosilicate, Polyacrylic acids; UDMA
VITREMER	3M Dental Products. St-Paul, MN, USA	Vitremer Primer: HEMA, Vitrebond Copolymer, Ethanol Photoinitiator	VITREMER: Fluoroaluminosilicate, Polymethacrylic acids, Ascorbic acid, HEMA
PHOTAC-FIL	ESPE GmbH, Seefeld, Germany.	No Primer	PHOTAC-FIL: Fluoroaluminosilicates, Sodium, Calcium, Polyacrylic acids, Maleic acid, HEMA

* Composition according to manufactures' information.

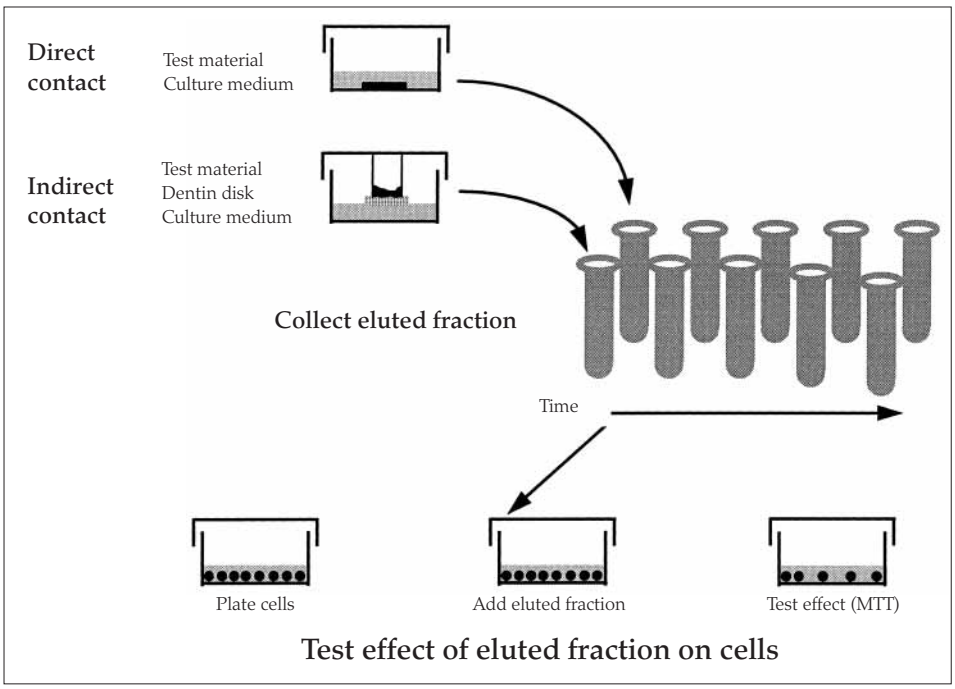

Fig. 1 Diagram of the experimental set-up used to test cytotoxicity of resin-reinforced glass ionomer cements. For the direct technique, components of the primers were directly extracted into the culture medium. For the indirect technique, the material was applied to dentin, and the disk placed in contact with 1 mL of cell-culture medium in the well below. Negative controls had no material. The medium were changed at 24 h intervals through 7 days and then tested for cytotoxicity using the MTT assay.

For the Dyract-Prime & Bond 2.0 system (De Trey-Dentsply, Konstanz, Germany) two successive coats of Prime & Bond 2.0 were brushed onto the moist dentin and left undisturbed for 30 seconds before evaporating the solvent with a dental air syringe. The resin was light cured for 20 seconds and a two millimeters thick layer of Dyract restorative material was further polymerized for 40 seconds. The same protocol has been used for the Dyract-Prime & Bond 2.1 system (De Trey-Dentsply). For the Vitremer Tri Cure Glass Ionomer System (3M St Paul, MN, USA), the primer was applied on the moist dentin during 30 seconds, gently blown with an air syringe and photopolymerized for 20 seconds. The Vitremer powder and liquid were mixed homogeneously and introduced within a 2 mm thick layer in the polystyrene tube previously glued to the dentin disk. It was further light cured during 40 seconds. The Photac-fil Applicap material (ESPE, Seefeld, Germany) was mixed for 15 seconds and applied directly on dentin without using a priming system. Polymerization of the material was achieved by light curing during 40 seconds.

Phenol was used as positive control because it can diffuse through dentin and elicit toxicity to cultured cells (HANKS et al. 1989). A saturated solution of 0.7 M phenol was prepared by dissolving phenolic salt into phosphate buffer saline solution and placed on the top of the dentin disks. Negative controls consisted of dentin disks without the application of glass ionomer cements. There were eight replicates for each condition. Then, each specimen was placed in contact with 1 mL of cell culture medium (DMEM) and incubated at 37° C. Only the pulpal side of the dentin disk came into contact with the cell culture medium so that only diffusates through dentin were extracted with the collection medium. Every 24 hours, the medium was collected and replaced with fresh medium for another 24 hours. This procedure was repeated every 24 hours during the seven days of the experiment (Fig. 1).

The cytotoxicity of the cell-culture medium eluates was then assessed. Human fibroblasts were grown from the pulp of non apexified wisdom teeth surgically extracted (CHRISTEN et al. 1989). Cells were grown to confluence at 37° C, 5% CO_2, and 95% humidity and were subcultured in DMEM supplemented with 5% foetal calf serum, 2% L-glutamin and 1% penicillin-streptomycin (all from Seromed, Basel, CH). This guaranteed that cultures would have a sufficient number of cells to assess the cytotoxicity of all eluates using the same crop of cells in the 3rd passage. For experiments, cells were plated at 25,000 cells / cm^2 in 96-well plates and were incubated for 24 h before the addition of the eluates. The culture medium was then removed and 0.2 mL of eluate was added to each well for 24 h (Fig. 1). The succinic dehydrogenase activity (SDH) of the monolayers was assessed using the MTT method previously described by MOSMANN (1983). Briefly, after exposure to the eluates of the glass ionomer cements, the medium was replaced by 0.1 mL of MTT solution and incubated for one hour at 37° C. The SDH activity was quantified by dissolving the MTT-formazan in a solution of 0.1 N NaOH (6.25%) in dimethyl sulfoxide, and reading the absorbance of the resulting solution at 550 nm. Statistical differences were assessed by ANOVA and Student Newman Keuls tests ($P < 0.05$).

Results

Results for the cytotoxicity of the primers placed in direct contact with cell monolayers are presented in Figure 2. The three primers tested elicited comparable cytotoxicity as demonstrated by the reduced enzymatic activities relative to control cultures without materials. After exposure to 24 and 48 hours extracts, SDH activities of the cells were significantly depressed ($P < 0.05$) by 35%. For all materials, metabolic activities gradually increased for each period of time until 5 days and remained approximately constant for the 5 and 6 days extracts. After exposure to the seven days extracts suppression levels slightly increased for the Prime & Bond 2.0 and Prime & Bond 2.1.

Eluates from glass ionomer cements collected after diffusion through dentin affected the SDH activity of the cells, but not equally for all materials. The results are presented in Figure 3 and expressed as a percentage of the mean enzymatic activity measured for the cells exposed to the dentin disk without material (negative control). When applied to dentin all materials elicited cytotoxicity as demonstrated by the reduction in enzymatic activities of the cultured cells. After exposure to 24 hours diffusates, SDH activities of the cells were depressed to 31%, 82%, 89% and 93% for respectively the Vitremer Tri Cure System, the Dyract-Prime & Bond 2.0, the Dyract-Prime & Bond 2.1 and the Photac-Fil. After 24 hours, metabolic activities gradually increased for each period of time and were not statistically different from the control except for the cells exposed to eluates from Vitremer. For the Vitremer extracts, SDH activity remained significantly depressed ($P < 0.05$) by almost 20% through seven days. When measuring the MTT activity after exposure to eluates from phenol, results showed that early extracts (1 to 4 days) statistically reduced SDH activity below control level ($P < 0.05$). After 4 days, the SDH activities of cells exposed to phenol remained constant and similar to negative control.

Discussion

The current study was designed to assess the possibility that leachables from modern glass ionomer cements might diffuse through dentin and cause cytotoxic effects on pulpal cells. Results for the primers placed in direct contact with the cells showed a higher toxicity with early extracts (Fig. 2). This result can be attributed to the initial release of unreacted monomers in

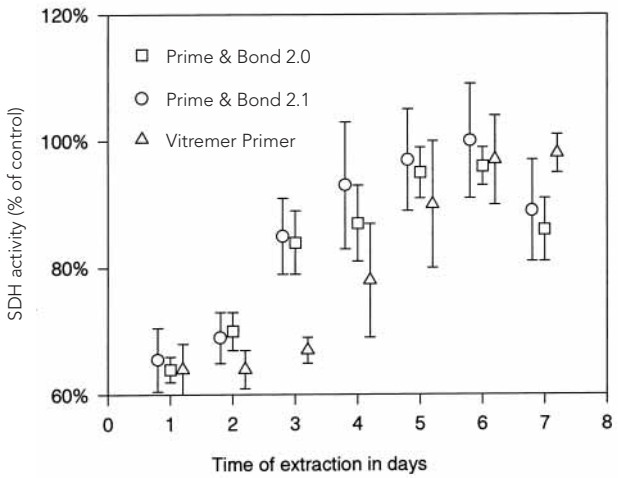

Fig. 2 Succinic dehydrogenase (SDH) activity of cells placed in contact with one day to seven days extracts of the three different primers tested. Eluants were in contact with human pulpal fibroblasts for 24 h. SDH activity was expressed as a percentage of controls without material (100% = no effect). Error bars indicate standard deviations of eight replicates for each material.

the wet environment (FERRACANE & CONDON 1990). Nevertheless, cell metabolism was no longer affected by the 5 and 6 days extracts and the incorporation of fluoride into the Prime & Bond 2.1 did not markedly affect the biological properties of this resin compared to the original formulation (Prime & Bond 2.0). Finally, it must be noted that the slight increase in toxicity observed for the seven days extracts could be related to the dissolution of the primers into the culture medium. This phenomenon was clearly observed at the end of the experiment with the three primers tested.

Although the dynamics of the pulpodentinal complex cannot be reproduced under *in vitro* conditions, results from the indirect technique probably reflect better the risk for pulpal irritation when resin-modified glass ionomer cements are used. As reported by HANKS et al. (1994), dentin can adsorb substances in the tubules and limits the diffusion of noxious substances into the pulp. Previous *in vitro* studies have demonstrated a reduced apparent cytotoxicity of diffusates when the materials are separated from cells by a dentin barrier (HANKS et al. 1996). This could explain the favorable results reported from the current study. Further, the 1 mL of collection medium probably diluted the toxical components that diffused through the dentin but the results for phenol confirmed that this model was successful in evaluating the toxicity of the diffusates. Finally, several *in vitro* studies used experimental models where toxicants were diluted by a volume of 4 mL which is a much greater volume than that occurring *in vivo* (MERYON 1984, HUME & MOUNT 1988). Although the composition of each material was clearly a factor in the amount of cytotoxicity, the exact relationship between the observed effects and the concentration of the diffused components cannot be established since the composition of each material is proprietary. Nevertheless, the strong cytotoxic effects observed in the presence of the 24 hours diffusates from Vitremer could be attributed to a rapid diffusion of hydrophilic components such as HEMA (Fig. 3). Recently, HEMA has been shown to diffuse rapidly across dentin because of its small molecular weight and high water solubility (BOUILLAGUET et al. 1996). However, the concentrations of HEMA that can diffuse into pulpal tissue fluids are significantly below those causing acute toxicity. Therefore, the risk that other molecules have been solubilized by the HEMA cannot be ruled out. This hypothesis is in agreement with the results of RATANASATHIEN et al. (1995) that demonstrated the additive cytotoxic effects produced by HEMA when used as a solvent for BIS-GMA. Therefore, for the Vitremer, the leaching of a combination of toxic components including resin monomers and fluoride ions can be suspected. Recently, KAN et al. (1997) have reported a correlation between cytotoxicity and fluoride release of modern glass ionomer cements. They also pointed out that the leaching of other unidentified toxic components may have contributed to the toxicity of these new materials. Further, FRIEDL et al. (1997) have demonstrated that over a period of two weeks, Vitremer can release significant amounts of fluorides compared to Dyract. These previous studies support the results reported for the Vitremer in the current study. However, the incorporation of resins such as TEGDMA or fluorides (Prime & Bond 2.1) into the primers of the Dyract system did not markedly affect the biological properties of this material (Fig. 3). Although the toxicity of the TEGDMA has been reported (RATANASATHIEN et al. 1995), the low water solubility of this resin probably prevented its diffusion through dentin into the cell culture medium (HANKS et al. 1994). Further, FRIEDL et al. (1997) demonstrated that the amounts of fluorides released from resin-reinforced glass ionomer cements are significantly lower than those released from conventional glass ionomer cements. These observations could partially explain the favorable results reported for the Dyract system. Finally, the low toxicity reported for the Photac-Fil material could be attributed to the conservation of the smear layer in absence of acidic primer. As previously reported, the presence of a smear layer reduces the permeability of the dentin and subsequently the amount of toxic components that can reach the pulpal space (BOUILLAGUET et al. 1998). Further, for the Photac-Fil material, the absence of acidic primer totally prevented the risk of diffusion inside dentinal tubules of free, unpolymerized monomers that usually occurs during primer application. This hypothesis is also supported by the results reported for the Vitremer primer which was less toxic in direct contact after a carefully controlled polymerization (Fig. 2), than when applied through dentin where the polymerization remains uncertain (Fig.3). As a result, a particular attention should be paid during the polymerization of resin primers to reduce the risk of pulpal irritation under resin-reinforced glass ionomer cements.

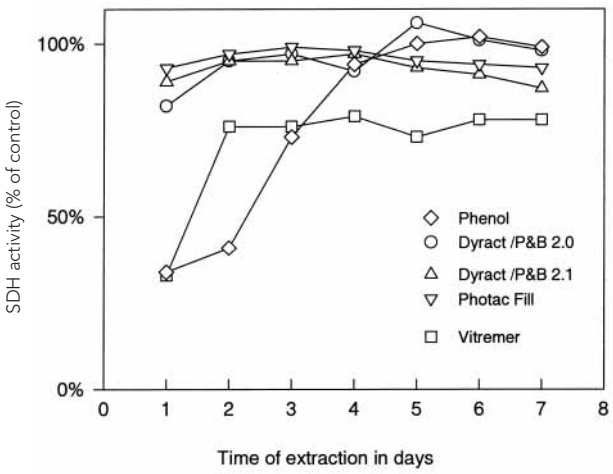

Fig. 3 The effect of time interval on metabolic activity (SDH) of cells placed in contact with diffusates through dentin of four resin reinforced glass ionomer cements. Eluants were in contact with human pulpal fibroblasts for 24 h. SDH activity was expressed as a percentage of controls without material. Error bars are not shown to avoid clutter, but all errors were of similar magnitude as in figure 2.

Conclusion

It can be concluded that most materials released a sufficient amount of leachables to elicit toxicity to the cultured cells. Materials were most toxic at early intervals and were generally less cytotoxic at later intervals although there were exceptions. This apparent toxicity could be attributed to the leaching of resin monomers and fluoride ions from the materials. The results suggest that the application to dentin of Vitremer poses a potential risk for pulpal irritation whereas the other resin-reinforced glass ionomer cements tested exhibited favorable biological properties. Further *in vivo* studies are required to confirm these *in vitro* results.

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Résumé

L'étude *in vitro* visait à comparer la cytotoxicité envers les cellules pulpaires des agents de liaison et des produits de diffusion dentinaire de quatre ciments aux verres ionomères modifiés (Vitremer Tri Cure System, Dyract-Prime & Bond 2.0, Dyract-Prime & Bond 2.1 et Photac-Fil). Les agents de liaison ont été placés directement dans le milieu de culture alors que les ciments ont été appliqués sur de la dentine humaine au contact du milieu de culture. Les milieux ont été collectés toutes les 24 heures pendant sept jours et incubés en présence des cellules pulpaires. La réponse cellulaire a été évaluée par la méthode du M.T.T. L'exposition des cellules aux produits de relarguage des agents de liaison a entraîné des altérations métaboliques sévères dans les premières 48 heures. Les effets cytotoxiques observés après exposition aux produits de diffusion dentinaire du Dyract-Prime & Bond 2.0, Dyract-Prime & Bond 2.1 et Photac-Fil étaient généralement plus sévères au début qu'à la fin du test. Pour le Vitremer, une réduction d'environ 20% de l'activité enzymatique était toujours observable au 7e jour. Si la majorité des ciments aux verres ionomères testés dans cette étude ont présenté des propriétés biologiques favorables, celles-ci doivent cependant être confirmées cliniquement.

Zusammenfassung

Frühere Studien ergaben, dass sich toxische Komponenten aus Glasionomerzementen lösen und durch die Dentintubuli in die Pulpa diffundieren können. Die vorliegende In-vitro-Studie untersuchte die zytotoxische Wirkung von löslichen Anteilen von Primer-Systemen sowie von durch Dentin diffundierbaren Stoffen aus vier Glasionomerzementen (Vitremer Tri Cure System, Dyract-Prime & Bond 2.0, Dyract-Prime & Bond 2.1 und Photac-Fil) auf Pulpafibroblasten. Die Primer wurden direkt mit Zell-Kulturmedium extrahiert, während die Glasionomerzemente auf menschliche Dentinscheiben aufgebracht und im darunter liegenden Kulturmedium aufgefangen wurden. Die Medien wurden während sieben Tagen alle 24 Stunden gesammelt und während 24 Stunden mit Pulpafibroblasten inkubiert. Die Reaktion der Zellen wurde mit der MTT-Methode gemessen. Die Primer zeigten in den ersten 24–48 Stunden den stärksten zytotoxischen Effekt. Die vier harzverstärkten Glasionomerzemente gaben genügend toxische Komponenten durch das Dentin hindurch ab, um den Zellstoffwechsel zu reduzieren. Im allgemeinen war die Wirkung am Anfang der Testserie stärker als am Ende, doch bei Vitremer wurde selbst am 7. Tag noch eine Reduktion des Zellstoffwechsels um ca. 20% beobachtet. Obwohl die Mehrzahl der Glasionomerzemente in dieser Studie sich durch niedrige Toxizität auszeichneten, müssen diese Ergebnisse noch klinisch bestätigt werden.

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