Titanium dioxide nanotubes added to glass ionomer cements affect S. mutans viability and mechanisms of virulence

Abstract: This in vitro study evaluated the impact of TiO$_2$ nanotubes (n-TiO$_2$) incorporated into glass ionomer cement (GIC) on Streptococcus mutans (S. mutans) characteristics at cellular and molecular levels. n-TiO$_2$, synthesized by the alkaline method (20 nm in size), was added to Ketac Molar EasyMix® at 0%, 3%, 5%, and 7% by weight. S. mutans strains were cultured on GIC disks with addition or not of n-TiO$_2$ for 1, 3, and 7 days and the following parameters were assessed: inhibition halo (mm) (n=3/group); cell viability (live/dead) (n=5/group); cell morphology (SEM) (n=3/group); and gene expression by real-time PCR (vicR, covR, gtfB, gtfC, and gtfD) (n=6/group). The data were analyzed by the Kruskal-Wallis test, repeated-measures ANOVA or two-way ANOVA, and Tukey’s and Dunn’s post-hoc tests (α=0.05). The agar diffusion test showed a higher antibacterial property for 5% n-TiO$_2$ compared with 3% and 7% (p<0.05) with no effect of time (1, 3, and 7 days). The cell number was significantly affected by all n-TiO$_2$ groups, while viability was mostly affected by 3% and 5% n-TiO$_2$, which also affected cell morphology and organization. Real-time PCR demonstrated that n-TiO$_2$ reduced the expression of covR when compared with GIC with no n-TiO$_2$ (p<0.05), with no effect of time, except for 3% n-TiO$_2$ on vicR expression. Within-group and between-group analyses revealed n-TiO$_2$ did not affect mRNA levels of gtfB, gtfC, and gtfD (p>0.05). Incorporation of n-TiO$_2$ at 3% and 5% potentially affected S. mutans viability and the expression of key genes for bacterial survival and growth, improving the anticariogenic properties of GIC.

Keywords: Nanotechnology; Glass Ionomer Cements; Titanium; Gene Expression.

Introduction

Glass ionomer cement (GIC) is a polyvalent restorative material based on an acid-base reaction between a powder (silicate, fluoride, or aluminum) and a liquid (polyacrylic, maleic, tartaric, or itaconic acids)\(^1\). The use of GIC in clinical dentistry is associated with its chemical bonding to the dental structure and to its anticariogenic activity as a result of ion release.\(^2\) By contrast, GICs are fragile in highly tense regions because of their low cohesive strength,\(^3\) especially in multiple-surface cavities.\(^4\) Furthermore, the fluoride content released from GIC varies from 2 to 10 ppm in the first
Titanium dioxide nanotubes added to glass ionomer cements affect S. mutans viability and mechanisms of virulence

2 days\(^5,6\) and this release dramatically decreases up to 14 days.\(^7\) Thus, the use of GIC is frequently limited to atraumatic restorative treatment (ART), procedures in primary teeth,\(^4\) and non-carious cervical lesions.\(^3,10\)

In order to improve the anticariogenic activity and mechanical properties of GICs, some studies have focused on the addition of bioactive particles to ionomer materials.\(^11,12,13\) Among several materials, titanium dioxide (TiO\(_2\)) has been incorporated into GIC\(^14,15,16,17\) due to its high biocompatibility and mechanical properties and also because of its potential antimicrobial activity. Recent studies have shown that GIC reinforced with nanostructured TiO\(_2\) exhibited improved mechanical properties,\(^15\) increased fluoride release,\(^14\) and a higher antimicrobial potential compared with conventional GIC.\(^16,17\) However, the mechanisms associated with the antimicrobial effects of TiO\(_2\) nanotechnologies remain unclear. Furthermore, some authors support that size, shape, and crystallinity are determining factors for the final quality of titanium nanotubes (n-TiO\(_2\)).\(^18,19,20\) It has been suggested that the effect of nanoparticle incorporation into GIC is potentially related to the improvement of homogeneity and consistency of the material, which reduces microcracks and air void formation into the cement matrix.\(^21\) Therefore, the use of nanotechnology represents a remarkable advance in developing biologically active materials.\(^22\)

Apart from that, antibacterial agents incorporated into restorative materials have suppressed bacterial growth,\(^23,24,25\) helping minimize the risk of recurrent caries and damage to the dental pulp.\(^2\) The incorporation of antibacterial agents possibly affects the structure and metabolism of bacteria by damaging important genes\(^25\) such as glucosyltransferases (gtfB, gtfC, and gtfD), responsible for extracellular polysaccharide synthesis, and the two-component transduction systems (TCS), corR and vicR, which are, respectively, inhibitory and excitatory response regulators for bacterial virulence that modulate the expression of gtf genes.\(^26,27\)

However, it is not known whether n-TiO\(_2\) addition to conventional GIC would affect cellular and molecular mechanisms involved in bacterial cariogenic potential. Therefore, the aim of this in vitro study was to assess the impact of different concentrations of n-TiO\(_2\) incorporated into GIC on the biology of S. mutans cultures at the cellular and molecular levels. In the present investigation, the null hypothesis was that n-TiO\(_2\) added to a conventional GIC would not affect S. mutans biology during the initial periods of biofilm formation, including cell viability, morphology, and gene expression.

### Methodology

#### Experimental design

The current study assessed the incorporation of TiO\(_2\) nanotubes (n-TiO\(_2\)) based on concentration levels (% by weight) into a conventional high-viscosity GIC (Ketac Molar Easymix™, 3M/ESPE, Maplewood, Minnesota, USA, batches #585454, #635287, #610966, and #588869) and the maturation time of the single-species biofilm formed on the material (1, 3, and 7 days). GIC samples were assigned to four experimental groups according to the concentration of n-TiO\(_2\): GIC = Control; GIC+3% n-TiO\(_2\); GIC+5% n-TiO\(_2\); and GIC+7% n-TiO\(_2\) following the previous studies of our group.\(^14,15\) S. mutans strains were cultured on GIC disks and the following parameters were assessed: inhibition halo (mm) (n=3/group); cell viability – live/dead (n = 5/group); cell morphology (SEM) (n = 3/group); and gene expression (real-time PCR) (n = 6/group). Experiments were done in triplicate and repeated at least twice, according to ISO 10993-5 (2009) recommendations.\(^28\)

#### Materials

n-TiO\(_2\) (particle size ≈ 20 nm and diameters ≈ 10 nm) was synthesized by the alkaline route.\(^29\) Briefly, n-TiO\(_2\) was prepared by mixing 12 g of TiO\(_2\) anatase phase 99% purity with 200 mL of 10 M NaOH. This mixture was kept at 120°C for 24 h in an open Teflon vessel, which was placed in a glycerin bath, using a mantle heater. The syntheses were carried out at ambient pressure, where only precursor reagents were subjected to alkaline treatment. After alkaline treatment, the mixture was washed with 0.1 M hydrochloric acid and deionized water repeatedly to remove sodium ions. The pH of the solution was then adjusted to 7. Finally, the materials obtained were dried in a conventional oven at 200°C for 24 h.\(^14,29\)
conventional GIC, Ketac Molar EasyMix™ [shade A3; powder: Al-Ca-La fluorosilicate glass, 5% copolymer acid (acrylic and maleic acids) (15 g); liquid: acrylic acid-maleic acid copolymer (25-40% by weight), tartaric acid (5-10% by weight), and water (10 g)] was used based on a previous review. 4

**GIC sample preparation**

n-TiO$_2$ was weighed in a precision scale with 0.0001 g readability (Adventurer Ohaus, Parsippany, New Jersey, USA), added to the GIC’s component powder and homogenized vigorously in a QL-901 vortex mixer (Biomixer, Taft, USA) for 2 min. 14,15 The recommended powder/liquid (P/L) ratio of 1/1 for GIC was used for all prepared samples and manipulation was done following the manufacturer’s specifications. GIC and GIC-n-TiO$_2$ disks were prepared using Teflon bipartite disk molds in one increment and pressed for 6 min between the polyester matrix (Proben, Catanduva, Brazil, #PR5021) and glass plates. They were covered with a thin layer of petroleum jelly (Rioquímica, São José do Rio Preto, SP, Brazil, #1702146) and stored for 24 h at 37°C and 100% humidity. The disks were exposed to UV light for 15 min for each surface before the experiments. 14

**Streptococcus mutans cultures**

*S. mutans* UA159 (ATCC 25175) strains were used in the present study. For each experiment, 300 µL of the frozen stock were freshly cultured in 15-mL Falcon tubes with 5 mL of brain-heart infusion (BHI) broth (DIFCO Laboratories, Detroit, MI, USA). The absorbance of 0.135 at 660 nm was achieved to obtain a concentration of 1x10$^8$ cells/mL (Genesys 2 spectrophotometer, Spectronic Unicam, Waltham, USA). 30 The cultures were incubated for 24 h at 37°C in 10% CO$_2$ for the subsequent experiments.

**Agar diffusion test**

A base layer containing 15 mL of sterile BHI medium mixed with the inoculum was prepared for each Petri dish (15 x 90 mm). After BHI agar solidification, six wells measuring 5 mm in diameter were prepared in each plate and filled with one of the experimental materials (GIC with or without n-TiO$_2$). All materials were handled under aseptic conditions according to the manufacturer’s instructions and inserted into the wells using a syringe (Centrix Inc., Shelton, USA). A thin layer of the agar was added to the wells, allowing total incorporation of the material in the culture medium. Chlorhexidine digluconate 0.12% solution and sterilized deionized water (10 µL) were applied on sterile filter paper disks as positive and negative controls, respectively. The dishes were kept for 2 h at room temperature to allow the diffusion of the materials and were then incubated at 37°C and 10% CO$_2$. After 1, 3, and 7 days, inhibition zones around the materials were measured (mm) using a digital caliper (Mitutoyo, MTI Corporation, Tokyo, Japan) by one calibrated evaluator (Spearman’s correlation = 87%).

**Cell viability test (live/dead)**

*S. mutans* strains were cultured on GIC disks (2 mm in height x 4 mm in diameter) in 24-well plates for 1, 3, and 7 days (37°C and 10% CO$_2$). The culture medium was then aspirated and the disks gently washed with saline solution. The plates were then maintained in a dark room and 25 µL of a live/dead Baclight bacterial viability staining solution was used (Molecular Probes, Eugene, USA). The excitation/emission wavelengths of SYTO9 and propidium iodide were 480/500 nm and 490/635 nm, respectively. Six images were captured under a fluorescence microscope (Zeiss, Jena, Germany) at 400X magnification from randomly selected sites for each analyzed surface. To determine the viability of the adhered bacterial species for each type of surface treatment, the green and red zones were separately determined, representing live and dead bacterial cells, respectively. The bacterial cell count for each dye in relation to the total area was performed on the ImageJ software (National Institute of Health, NIH, USA) and presented in arbitrary units (a.u.) and percentage for each surface. Positive and negative controls included bacteria cultured on glass coverslips and cultures treated with chlorhexidine digluconate 0.12% solution and plated on glass slides, respectively.
Titanium dioxide nanotubes added to glass ionomer cements affect S. mutans viability and mechanisms of virulence

Scanning electron microscopy analysis (SEM)

S. mutans strains were cultured on GIC and GIC-n-TiO₂ disks (2 mm × 4 mm) for 1, 3, and 7 days in 24-well plates in triplicate (Costar Corp, Cambridge, USA). After each experimental period, the cells were fixed in Karnovsky’s solution (2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) at room temperature for 2 h. Subsequently, the samples were dehydrated in a graded series of ethanol and then stored in an oven at 37 °C for overnight dehydration. They were then dried to a critical point (Denton Vacuum, mod. DCP-1, Moorestown, USA), coated with 10 nm of a gold layer on sputter coater (BAL-TEC, model SCD 050, Fürstentum, Liechtenstein) and kept in a desiccator until analysis. The structure and cell morphology of S. mutans colonies were assessed by a JEOL scanning electron microscope (JSM5600LV, Akishima, Tokyo, Japan) at 2000X magnification, 15 kV, and 10 mm of working distance.

Gene expression analysis by real-time PCR

S. mutans strains were cultured at 37°C and 10% CO₂ in 1.5 mL of BHI + 1% sucrose on GIC disks (2 mm × 8 mm) with or without the different concentrations of n-TiO₂ in 24-well plates for 24 and 72 h. The culture medium was replaced every 24 h with fresh sterile BHI + 1% sucrose. Gene expression assays were performed as previously described with some modifications. Briefly, 1 and 3 days after biofilm growth, the disks were removed from the culture medium and transferred to tubes containing 2 mL of saline solution (0.9% NaCl). The samples were vortexed (2800 rpm/10 s) to promote cell detachment from the surface of disks, and the cell-containing solution was transferred to 2 mL microtubes (Axigen, New York, USA) and centrifuged (2 minutes, 4 °C, ≈ 16000 g) for cell pellet precipitation. Pellets were stored at -80°C until use. Frozen cells were disrupted using 0.16 g of 0.1 mm diameter zirconia beads (Biospec, Bartlesville, USA), combined with 220 μL of TE buffer on a Mini-Beadbeater apparatus (Biospec), and total RNA was obtained using the RNeasy Mini Kit system as recommended by the manufacturer (Qiagen, Hilden, Germany). Next, 60 ng of total RNA was converted to cDNA using iScript Reverse Transcriptase (Bio-Rad Laboratories, Hercules, CA, USA) following the recommended manufacturer’s protocol. Real-time PCRs were performed on LightCycler 480 System (Roche Diagnostics GmbH, Germany) using specific primers for vicR, covR, gtfB, gtfC, and gtfD genes. Transcript levels were determined using the delta CT method (Table 1).

Statistical analysis

Data distribution and homoscedasticity were analyzed by the Shapiro-Wilk, Levene’s, and Hartley’s tests, respectively (p ≥ 0.05). Inhibition halo data were subjected to repeated-measures ANOVA followed by Tukey’s test for multiple comparisons, whereas Dunn’s test was used to assess statistical differences between the control and experimental groups individually. Besides, cell viability (viable, non-viable, and total bacteria) was transformed by Box-Cox and two-way ANOVA and Tukey’s tests were performed. Gene expression data on covR, vicR and, gtfC were analyzed by two-way ANOVA followed by Tukey’s test. gtfB and gtfD values presented non-homogeneous distribution and were subjected to the Kruskal-Wallis test. Statistical analyses were

| Primers (qPCR) | Sequence 5’–3’ (Forward/Reverse) | Product size |
|---------------|----------------------------------|-------------|
| 16S RNA       | CGGCAAGCTAATCTCTGAAA/GCCCCTAAAAGG TTACCTCA | 190 bp* |
| SMU.910 (gtfD) | TGATTCGTGGTATCGTCCTAA/GTTGAGACTTT CTTGGCTGCT | 199 bp* |
| SMU.1005 (gtfC) | ACACACCGCCACTGTTACT/AACGGTTTACCGC TTTTGAT | 161 bp* |
| SMU.1004 (gtfB) | CGAAATCCCAAATTTCTAATGA/TGTTTCCCCAACAGTATAAGGA | 197 bp* |
| SMU.1517 (vicR) | AGTGGCTGAGGAAAATGCTT/CATCACCTGACCTGTGTGGT | 163 bp* |
| SMU.1924 (covR) | ACGAAATATGGACGCAACAC/CAGAGATGGACGGTATGAA | 185 bp* |

*Data obtained from Stipp et al., 2013.27
performed using SPSS Statistics (version 21, IBM Statistics, New York, USA) and SAS System (ISAS Institute, Cary, USA), considering a significance level with $\alpha=0.05$.

**Results**

**Agar diffusion test**

Table 2 illustrates the findings for the agar diffusion test in *S. mutans* cultures. The between-group analysis showed that GIC with addition of 5% n-TiO$_2$ had a superior antimicrobial activity ($p < 0.05$) compared with 3% and 7% n-TiO$_2$ groups but similar to that of the control group ($p > 0.05$) at all time points. Additionally, within-group comparisons demonstrated no significant differences ($p > 0.05$) for antibacterial activity achieved at 24 h and at 3 and 7 days. Chlorhexidine, as the control of the method, presented the highest antibacterial capacity ($p < 0.05$).

**Cell viability**

Figure 1 shows representative fluorescence microscopy images illustrating the live/dead assay for all groups and time points. Note the presence of more red cells for 3% and 5% concentrations for the initial time points, and higher counts of non-viable cells at 7 days for all groups, mostly GIC and GIC+3% n-TiO$_2$. Figure 2 presents the total cell counts (%/area) and the viable and non-viable cell numbers. There was a significant difference for the investigated factors: n-TiO$_2$ concentrations ($p=0.0405$); time of bacterial growth ($p < 0.001$); and interaction between the two factors ($p<0.001$). The within-group analysis showed that GIC+7% n-TiO$_2$ reduced the number of total bacterial counts over time with a significant difference at 7 days, whereas GIC+3% n-TiO$_2$ displayed higher total bacterial counts at 7 days than at 3 days ($p < 0.05$). The number of viable cells was significantly lower for the control group at 7 days than at 1 and 3 days, and for GIC+7% n-TiO$_2$, compared with day 1 ($p < 0.05$).

The between-group analysis demonstrated an increased number of non-viable cells for all the experimental groups over time, with GIC+3% n-TiO$_2$ and GIC+5% n-TiO$_2$ displaying significant differences at day 7 ($p < 0.05$). The total number of cells was significantly reduced for the n-TiO$_2$ groups at day 1 as compared with the control group, whereas this trend was maintained at days 3 and 7 for the 3% and 7% n-TiO$_2$ groups, respectively ($p < 0.05$). The between-group analysis further revealed that the number of viable cells was significantly reduced for GIC+3% n-TiO$_2$ and GIC+5% n-TiO$_2$ at day 1 and GIC+3% n-TiO$_2$ at day 3, compared with the control group ($p < 0.05$), whereas the number of non-viable cells was not significantly affected ($p > 0.05$).

**Cell morphology by SEM**

Figure 3 illustrates SEM representative images for *S. mutans* cultured on GIC disks with addition or not of n-TiO$_2$ at 1, 3, and 7 days. SEM data showed that at 3% and 5%, the presence of n-TiO$_2$ affected cell morphology, resulting in a bacillary aspect with the cells organized in lines, whereas at 7% no visible cell morphology alteration could be noted.

**Table 2.** Agar diffusion test data illustrating the inhibition halo (mm) (means ± SD) produced by each material, including GIC with and without different concentrations of n-TiO$_2$, in cultures of *S. mutans* at 1, 3, and 7 days. Chlorhexidine (CHX) at 0.12% was used as a positive control.

| Experimental groups | 1 day       | 3 days     | 7 days     |
|---------------------|-------------|------------|------------|
| GIC (control)       | 8.49 (0.48)AAb | 8.74 (0.24) Aba | 8.41 (0.58) Aba |
| GIC+3% n-TiO$_2$    | 8.28 (0.46) Ba | 8.61 (0.29) Ba | 8.24 (0.40) Ba |
| GIC+5% n-TiO$_2$    | 8.77 (0.44) Aa | 8.84 (0.43) Aa | 9.06 (0.37) Aa |
| GIC+7% n-TiO$_2$    | 7.81 (0.60) Ca | 7.55 (0.68) Ca | 8.21 (0.50) Ca |
| CHX                 | 20.04 (0.48)* | 20.50 (0.46)* | 20.02 (0.65)* |

GIC: Glass ionomer cement; n-TiO$_2$: Titanium dioxide nanotubes; CHX: Chlorhexidine 0.12%. Different uppercase and lowercase letters represent between-group and within-group statistical differences, respectively, according to repeated-measures ANOVA followed by Dunn’s test for additional comparisons ($p < 0.05$). *Indicates differences in Dunn’s test considering chlorhexidine as the positive control ($p < 0.05$).
Gene expression analysis

Overall, the data analysis showed that covR was the most sensitive gene to the presence of n-TiO₂ in the GIC matrix, whereas neither time nor n-TiO₂ addition significantly affected transcript levels of vicR, gtfB, gtfC, and gtfD in S. mutans cultures (Figure 4). At 24 h, the presence of n-TiO₂ at 3%, 5%, and 7% significantly reduced the expression of covR as compared with GIC alone (p < 0.05) with the lowest mRNA levels for the 3% group. Similar findings were observed at 72 h, except for the fact that covR mRNA levels were similar to those of GIC alone (p > 0.05). In
addition, our findings demonstrated that, although not significant, vicR, gtfC, and gtfD mRNA levels tended to decrease over time regardless of n-TiO2.

**Discussion**

In the current study, the data analysis showed that n-TiO2 added to GIC potentially affected *S. mutans* properties. The presence of n-TiO2 led to increased antibacterial activity, affected bacterial growth and morphology, and altered the expression of *vicR*. By contrast, it did not affect mRNA levels of *gtfB*, *gtfC*, and *gtfD*. Previous studies have suggested a potential antibacterial effect of TiO2 nanostructures added to conventional GIC. However, caution should be exercised when comparing different studies, as distinct methodologies may have been used.

In addition to the agar assay, the potential antibacterial properties of n-TiO2 added to GIC were determined by assessing the impact of different concentrations of n-TiO2 on the cell viability and morphology of *S. mutans* cultures. Our findings demonstrated that GIC+3% n-TiO2 and GIC+5% n-TiO2 reduced *S. mutans* viability compared with the control group (GIC alone) and led to morphological alterations, including a rod-shaped structure rather than the typical spherical structure. These findings corroborate those of previous studies that reported antibacterial effects for n-TiO2. While the mechanisms for a potential antibacterial effect of n-TiO2 remain to be fully elucidated, it has been suggested that electrostatic interactions between metallic ions of nanostructured titanium and bacteria, attachment to the cell membrane, and the ensuing effects on phospholipids may explain some of the events controlling bacterial viability in the presence of n-TiO2. Furthermore, it has been suggested that specific physicochemical properties of nanostructures may play a critical role in their functionality and use. In this sense, the antimicrobial effects of n-TiO2 evidenced here could be associated with nanometric size, shape, and the crystallinity of its structure. TiO2 nanotubes used in this study had ~20 nm size...
Titanium dioxide nanotubes added to glass ionomer cements affect S. mutans viability and mechanisms of virulence

and ~10 nm diameter, which could facilitate their attachment to the cell membrane, inducing stress in the bacterial metabolism. This mechanism could be similar to that which occurs in other metallic nanoparticles. In addition, the anatase phase of TiO<sub>2</sub> nanotubes used in this study reacts with water to form a hydroxyl radical by photocatalysis, interfering with membrane phospholipids and causing damage to the bacterial DNA. Besides the potential direct effect of n-TiO<sub>2</sub> on bacterial survival as discussed earlier, we hypothesized that changes in the chemical properties of conventional GIC promoted by n-TiO<sub>2</sub> may also influence its antibacterial capabilities. GIC alone has the potential to inhibit bacterial growth, which is mainly explained by fluoride release. Nonetheless, n-TiO<sub>2</sub> has the potential to boost fluoride release by conventional GIC, which does not exclude the hypothesis that higher fluoride levels in the biofilm microenvironment may also play a role in bacterial viability rates modulated by GIC. In the current study,
gene expression was selected as the main variable of the study and it was used to calculate power and to determine sample size. Such an approach may have affected the statistical power of other variables including the number of cells and, therefore, these results must be interpreted with caution.

Moreover, we assessed whether n-TiO$_2$ added to conventional GIC would affect the expression of key genes controlling $S.~mutans$ virulence, including $vicR$, $covR$, $gtfB$, $gtfC$, and $gtfD$. Except for GIC+7% n-TiO$_2$ at 72 h, n-TiO$_2$ incorporated into GIC significantly decreased the expression of $covR$, whereas $vicR$, $gtfB$, $gtfC$, and $gtfD$ were not affected. Once $covR$ has been shown to play a critical role in regulating several virulence factors that can affect $S.~mutans$ physiology, we evidenced that GIC+3% n-TiO$_2$ and GIC+5%...
n-TiO₂ could interfere with biofilm formation,²⁶,³⁹ such as extracellular polysaccharide synthesis and interaction.²⁶ To the authors’ best knowledge, this is the first study that demonstrates an effect of n-TiO₂ on the expression of a key gene for bacterial survival and biofilm formation. Therefore, this study adds new evidence to the potential mechanisms involved in n-TiO₂ antibacterial effect. Since covR has been shown to negatively regulate the expression of gtfB and gtfC genes by directly binding to their promoter regions,²⁶ it could be expected that decreased levels of covR would lead to increased expression of gtfB and gtfC. By contrast, although n-TiO₂ at 3% and 5% increased mRNA levels for gtfB and gtfC, no statistical significance could be found. Thus, the expression of vicR for these groups has possibly worked as a counterpart to balance the expression of the gtfBC complex. This could be also assumed because cell viability for these groups was reduced and the cells were possibly trying to find mechanisms to preserve their viability. That assumption is also based on the expressions of gtfBC at the 7% concentration, which were more similar to the expected down-regulatory effect of covR, and on its number of viable and non-viable cells at 1 and 3 days. Together, these findings provide new insights into the potential mechanisms by which n-TiO₂ may affect bacterial metabolism in vivo, but future studies should be designed to determine the mechanisms by which TiO₂ affects the expression of key regulatory gene in S. mutans. Apart from that, it seems that GIC+3% n-TiO₂ has a progressive effect on S. mutans during the first 72 h, once vicR expression was lower at 72 h than at 24 h in the within-group comparison. Down-regulatory effects on vicR could affect cell membrane homeostasis since this gene is related to its biosynthesis,⁴⁰ corroborating the cell morphology modifications evidenced by SEM images. Importantly, a previous study has demonstrated that n-TiO₂ added to GIC had a positive impact on human cells (fibroblasts) and on cell morphology/spreading and ECM composition.¹⁴ Therefore, the present study demonstrates that the addition of n-TiO₂ to conventional GIC interfered with S. mutans metabolism, modulating the expression of key regulatory genes, affecting cell morphology and decreasing the number of viable cells. Although the use of nanotechnology has risen as a promising approach to modulate bacterial biofilms in vivo, at this time, the reported results refer to an in vitro monoculture system with no quantification of extracellular polysaccharides. Hence, future studies should be performed to determine whether n-TiO₂ also affects glucan synthesis and the matrix organization and to assess the effects of these materials on more complex multispecies biofilms in vivo.

**Conclusion**

The use of GIC added to n-TiO₂ at 3% and 5% can potentially affect bacterial biofilm formation by reducing cell viability and affecting the expression of key genes for bacterial survival and growth, improving the anticariogenic properties of GICs.

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