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***In Vitro* Evaluation of Planktonic Growth on Experimental Cement-Retained Titanium Surfaces**

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The purpose of this study was to compare the effects of selected cements, or their combination with titanium, on the growth of two periodontopathic bacteria: *Prevotella intermedia* (Pi) and *Fusobacterium nucleatum* (Fn).

Material/Methods: This study was comprised of several experimental groups: 1) Dental luting cements (glass ionomer cement, methacrylate-based resin cement, zinc-oxide eugenol cement, eugenol-free zinc oxide cement; 2) titanium discs; and 3) titanium combination cement discs. The disks were submerged in bacterial suspensions of either Fn or Pi. Planktonic bacterial growth within the test media was measured by determining the optical density of the cultures (OD₆₀₀). Mean and standard deviations were calculated for planktonic growth from three separate experiments.

Results: Intergroup comparison of all experimental groups revealed increased growth of Pi associated with cement-titanium specimens in comparison with cement specimens. Regarding the comparison of all groups for Fn, there was an increased amount of bacterial growth in cement-titanium specimens although the increase was not statistically significant.

Conclusions: The combination of cement with titanium may exacerbate the bacterial growth capacity of Pi and Fn in contrast to their sole effect.

MeSH Keywords: **Dental Cements • *Fusobacterium nucleatum* • Peri-Implantitis • *Prevotella intermedia***

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Background

Over the last few decades, osseointegrated dental implants have provided a high positive aesthetic impact and improvement in quality of life for completely and partially edentulous patients, substituting one or more missing teeth [1]. Prosthodontic procedures performed in connection with dental implants restore the functional and aesthetic requirements of the oral cavity with good occlusal harmony. Cement retention is mostly preferred in implant-supported fixed restorations versus screw retention due to relatively easier fabrication procedures and for cost containment issues. Dentists are also more familiar with cementation procedures [2].

Even though there is no consensus on a cement selection protocol for implant restorations [3], the cements designed for tooth-supported restorations are commonly used [4]. Although there are advantages in cement retention, the risk of leaving excess cement in the peri-implant sulcus is a possible cause for peri-implantitis when acting as a mechanical irritant or as a repository for bacteria or both [5–7]. Peri-implantitis is an inflammatory process that leads to the destruction of osseointegrated implant supported tissues such as alveolar bone. Although peri-implantitis is associated with a polymicrobial biofilm that is similar with the microflora in tooth sites, periodontopathogenic bacteria including *Porphyromonas gingivalis* (Pg), *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), *Aggregatibacter actinomycetemcomitans* (Aa) and *Fusobacterium nucleatum* (Fn) orchestrate progressive destruction and instigate inflammatory disease [8–11]. While some studies reported bacterial adherence to implant surfaces [10,12,13] and also documented implant complications as a result of cement extrusion into the subgingival peri-implant tissues [14,15], little is known about the influence of the type of excess cement on peri-implant disease progression.

Methacrylate cements may prepare a ground for the development of bacterial colonization and promote inflammation in the peri-implant tissue [16,17]. Furthermore, a clinical study on methacrylate cements supported this hypothesis where a methacrylate cement and a zinc oxide-eugenol cement were compared to investigate the clinical effect on the peri-implant tissue [18]. The results of the study showed that methacrylate cement led to more cement excess (62%), while excess cement was not detectable on any of the implant surfaces retained with a zinc oxide-eugenol cement. In addition, there were more signs of inflammation such as bleeding on probing and pocket suppuration in the methacrylate cement group.

Microbial colonization on the surfaces of dental implants causes infection of implant-supporting tissues. Periodontopathogenic bacteria, including Pg, Fn, Aa, Pi and Tf, have very similar effects on inflammatory peri-implant tissue disease to that

in periodontal disease of residual teeth [19]. Although excess cement around dental implants is reported as a risk factor for peri-implant disease by the American Academy of Periodontology [20], little is known about the influence of cement type on bacterial growth.

This study compared the effects of selected cements or their combination with titanium, on the growth of two periodontopathic bacteria: Pi and Fn.

Material and Methods

Specimen fabrication

The study was conducted with three different experimental groups: 1) dental luting cements; 2) titanium (Ti) discs; and 3) titanium combination cement disc group.

1) Dental luting cements

We used two permanent dental luting cements: glass ionomer cement (GI, Ketac Cem, 3M ESPE, MN, USA), and methacrylate-based resin cement (DT, DentoTemp, Itena, Paris France); and two temporary dental luting cements: zinc-oxide eugenol cement (E, Temp-Bond Original, Kerr, CA, USA), and eugenol-free zinc oxide cement (NE, Temp-Bond Non-Eugenol, Kerr, CA, USA).

Five stainless steel discs were machined to be 5 mm in diameter and 3 mm in thickness. The discs were bonded to a glass slab with wax boxing, and a single-mix impression of these discs was made with a polyether impression material to fabricate a mold. The cements were prepared in accordance with the manufacturer's instructions under aseptic conditions, dispensed into the mold and pressed between two glass plates. The glass plate was separated after the cement was set and disc shaped specimens (n=18) were produced for each cement with the same procedure. The cement specimens were sterilized with ultraviolet light [21].

2) Titanium discs

Titanium Grade 5 (TiAl₆V₄) rods (Bag-San, Istanbul, Turkey) 5 mm in diameter and 1 mm in length were sectioned into disc specimens of 3 mm thickness, using a lathe tool and (n=90) titanium specimens in total were obtained. The titanium specimens were subjected to long autoclave sterilization for 121°C/20 min.

3) Titanium combination cement discs

Seventy-two of 90 specimens titanium specimens were randomly selected and divided into four subgroups. In each subgroup (n=18), one of the diametral surfaces of the specimens

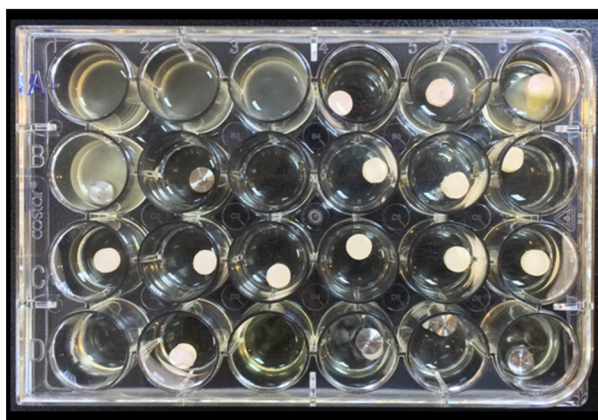


Figure 1. Tested well plate example after anaerobic incubation for determining planktonic growth of bacteria.

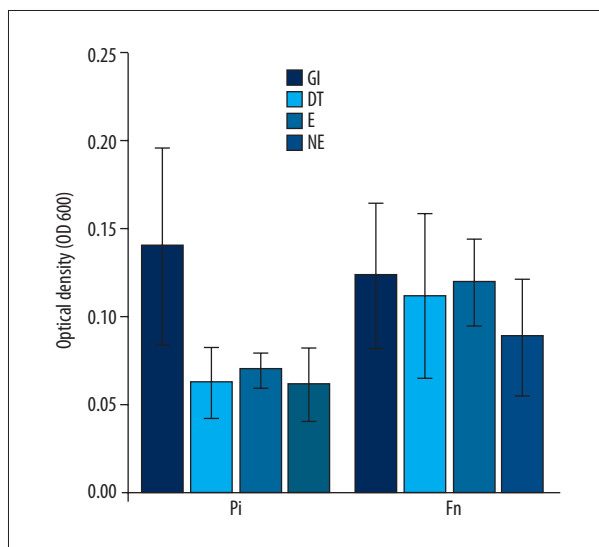


Figure 2. Planktonic growth measurement by OD₆₀₀ test values for *Prevotella intermedia* and *Fusobacterium nucleatum* in the presence of different cement disks. No significant difference was observed. GI – Glass Ionomer; DT – Dentotemp; E – Temp-Bond Eugenol; NE – Temp-Bond Non-Eugenol.

were covered with a thin layer of tested cements using a hand instrument. The cement specimens combined with titanium were also sterilized with U.V. [21]. The remaining 18 specimens out of 90 were used for the titanium group.

Bacterial strains and culture conditions

This study used Pi and Fn (strains isolated from clinical samples and stored at institute’s culture collection). Pi and Fn were grown in Brucella broth containing haemin (10 µg/mL), and menadione (5 µg/mL) in anaerobic conditions (5% H₂, 5% CO₂, and balance N₂) at 37°C for 24–72 h (Figure 1). The concentration of bacteria within the broth was estimated by

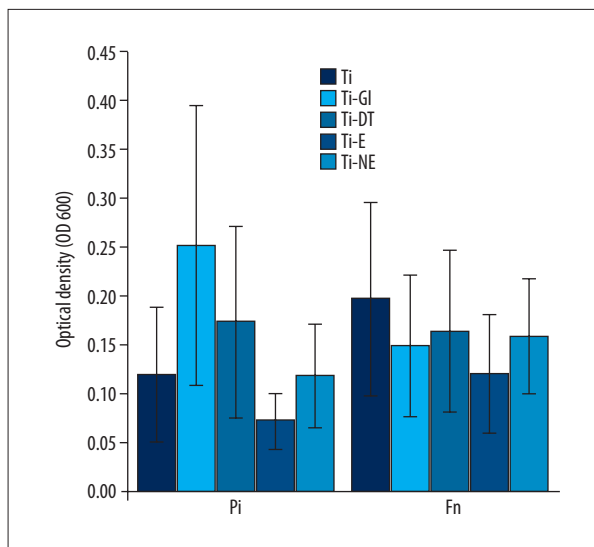


Figure 3. Planktonic growth measurement by OD₆₀₀ test values for *Prevotella intermedia* and *Fusobacterium nucleatum* in the presence of titanium combined different cement disks. Ti-GI displayed the most bacterial growth but the difference was not statistically significant in Pi. Ti – Pure Titanium disc; Ti-GI – Glass Ionomer combined with titanium; DT – Dentotemp combined with titanium; E – Temp-Bond Eugenol combined with titanium; NE – Temp-Bond Non-Eugenol combined with titanium.

measuring optical density (OD) at 600 nm in a spectrophotometer (Densichek, bioMérieux, France). OD₆₀₀ measurement directly correlates with the concentration of bacteria in a liquid culture.

Analysis of planktonic growth by spectrophotometer

Planktonic bacterial growth was determined by using a microplate reader (PR4100, Biorad, USA) as described in [22]. Cement, titanium and cement combined with titanium specimens were placed in individual wells of sterile 24-well plates and submerged in 1 mL liquid bacterial culture containing 10⁷ CFU/mL of tested bacteria (Figure 1). All types of materials in a sterile liquid broth served as a control to confirm that the cement specimens were not contaminated during experiments. Sterile liquid broth alone served as the negative control. Bacterial planktonic growth was determined by measuring the OD₆₀₀ of the bacterial culture from each test well after incubation under anaerobic conditions at 37°C for 48 h. All experiments were performed at least twice for verification, each time with a freshly prepared medium and subcultured bacterial strains.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 (IBM SPSS, Turkey) program. A Kruskal Wallis test was used for

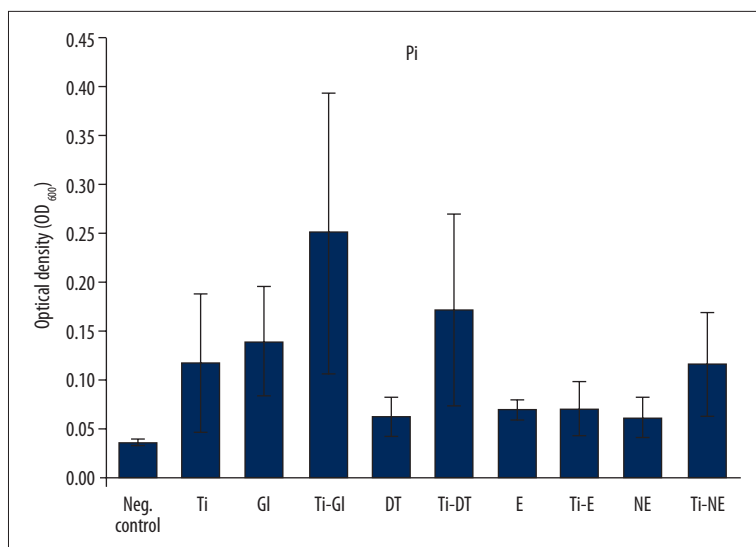


Figure 4. Planktonic growth measurement by OD₆₀₀ test values for *Prevotella intermedia* in the presence of cements and different cement discs combined with titanium. There was not significantly different among all groups. Ti – Pure Titanium disc; GI – Glass Ionomer; DT – Dentotemp; E – Temp-Bond Eugenol; NE – Temp-Bond Non-Eugenol Ti-GI – Glass Ionomer combined with titanium; DT – Dentotemp combined with titanium; E – Temp-Bond Eugenol combined with titanium; NE – Temp-Bond Non-Eugenol combined with titanium.

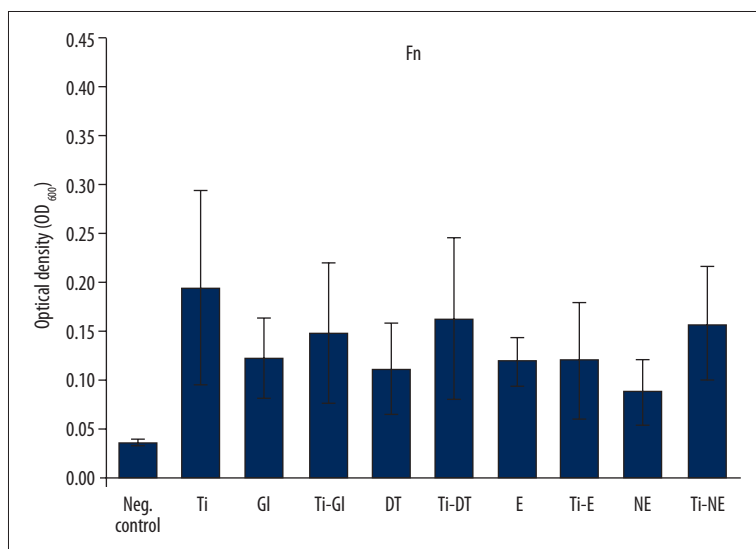


Figure 5. Planktonic growth measurement by OD₆₀₀ test values for *Fusobacterium nucleatum* in the presence of different cements and cements combined with titanium. There was not significantly different among all groups. Ti – Pure Titanium disc; GI – Glass Ionomer; DT – Dentotemp; E – Temp-Bond Eugenol; NE – Temp-Bond Non-Eugenol; Ti-GI – Glass Ionomer combined with titanium; DT – Dentotemp combined with titanium; E – Temp-Bond Eugenol combined with titanium; NE – Temp-Bond Non-Eugenol combined with titanium.

the intergroup comparisons of parameters. Significance was evaluated at a level of $p < 0.05$.

Results

Planktonic bacterial growth of Pi and Fn in each cement test group as determined by the OD₆₀₀ of each sample well are shown in Figure 2. The levels of bacterial growth for both Pi and Fn presented similar levels in all cement groups. GI presented the most prominent bacterial growth in culture media (Figure 2). Although there was no significant difference in bacterial growth among all cement specimens, NE displayed lower Pi and Fn levels in comparison with E for temporary luting cements, whereas DT showed the same effect for permanent luting cements (Figure 2).

After demonstrating the bacterial growth of cements in different culture media, the effect of cements combined with titanium on planktonic bacterial growth was investigated. According to the spectrophotometer values for Pi, titanium specimens showed less bacterial growth compared to all cement-titanium groups, except for Ti-E. Ti-E displayed the least bacterial growth, whereas Ti-GI displayed the most. The difference, however, was not statistically significant (Figure 3). In contrast, for Fn, titanium specimens inhibited the least bacterial growth compared to cement-titanium groups. Similar with Pi groups, Ti-E had the most inhibitory effect on bacterial growth of Fn (Figure 3). The intergroup comparison of all experimental groups revealed increased growth of Pi associated with cement-titanium specimens in comparison with cement specimens (Figure 4). Regarding the comparison of all groups for Fn, there was an increased amount of bacterial growth in cement-titanium specimens although the increase was not statistically significant (Figure 5).

Discussion

Gram-negative anaerobic bacteria are frequent pathogen findings in peri-implant diseases and other oral infections such as periodontitis. Although oral gram-negative microbiota exhibit a broad heterogeneity, both Pi and Fn can be isolated in the patients with peri-implantitis. *Provetella intermedia* is indole-positive and moderately saccharolytic [23] and commonly produces immunoglobulin-degrading enzymes [24], whereas Fn is a filamentous bacteria that produces serine protease capable of degrading extracellular matrix proteins [25]. As such, they can present different virulence and also growth characteristics as observed in the present study. *Fusobacterium nucleatum* is the first colonized anaerobic species of the mouth, which makes it the most common bacterial species in the mouth [26]. In this study, it was observed that Fn grew easier and also presented more consistent growth than Pi in an *in vitro* planktonic bacterial growth model.

The risk factors for peri-implantitis are classified as poor oral hygiene, prior history of periodontitis, habits like smoking, systemic disease that makes host susceptible to inflammation, inaccurate surgical procedures, and improper prosthetic rehabilitation [1]. Additionally, excess cement is reported as one of the risk factors for inflammatory peri-implant disease [27]. However there is still no consensus about the impact of the cement type on microbial colonization and biofilm formation on peri-implant tissue infection. In a retrospective clinical study, implant restorations cemented with methacrylate-based resin cement exhibited higher inflammatory periodontal indices (bleeding on probing, pocket suppuration and alveolar bone loss has been reported as higher than on implants cemented with a zinc-oxide eugenol cement [18].

An *in vitro* study where five different luting cements of zinc oxide-eugenol, eugenol-free zinc oxide, zinc orthophosphate, and two methacrylate-based resin cements were compared in terms of their effect on bacterial growth and biofilm formation associated with peri-implantitis, zinc-oxide cements presented lower growth levels of Aa, Pg, and Fn than methacrylate-based resin cements [22]. In accordance with the aforementioned results, the planktonic growth for both Pi and Fn in the present study, were lower in the presence of zinc-oxide cement although it was not statistically significant. In contrast, both bacteria presented higher growth levels in the presence of a glass-ionomer cement.

This comparative study investigated peri-implantitis associated bacteria, Pi and Fn, which are known as late colonizers [10,11]. To the best of the author's knowledge, this was the first study investigating the planktonic growth of these bacteria not only with cements, but also cements combined with titanium in an *in vitro* model. This experimental design was aimed at constituting the natural environment of a peri-implant region that represented peri-implant sulcus with luting cement residue

on a titanium abutment surface and its microbiological dynamics. No significant differences in growth of both Pi and Fn were observed among cement-titanium groups, although a decrease was seen in Ti-E cement disks. This may be attributed to the antibacterial activity of zinc-oxide nanoparticles with or without eugenol [28].

Pi growth was enhanced in the presence of Ti-GI. In an *in vitro* study, glass ionomer cement inhibited the growth of *Streptococcus mutans* and *Actinomyces viscosus*, whereas it had no antibacterial effect on *Enterococcus faecalis* [29]. The antibacterial effect of the cements may be specific to bacteria. For the titanium specimens, Fn demonstrated higher growth levels when compared to cement groups. Both Fn and Pi displayed more increased growth in cement groups than compared to cement-titanium groups. Sanchez et al. (2014) compared three different surface materials of dental implants, hydroxyapatite, titanium and zirconium, in an *in-vitro* biofilm model [30]. They demonstrated that the number of bacteria on hydroxyapatite was significantly higher than titanium and zirconium surfaces, but there was no difference between titanium and zirconium surfaces. They also reported that Fn reached its peak at 24 h whereas *V. parvula*, Aa and Pg reached a peak at 72 h on titanium surfaces, suggesting that each bacteria can present diverse growth behavior in different conditions. This in agreement with our data showing different growth levels of Pi and Fn with a titanium material.

This study was done with four different luting cements and with only two specific bacterial species, and as such, it does not fully represent the complex microbiota of peri-implant diseases. In addition, implant restorations in the oral environment are subjected to saliva containing various proteins and enzymes, food products and beverages, great extremes of temperature and functional or parafunctional loading. Therefore, a comprehensive evaluation of these factors may be considered for future research.

Conclusions

Within the limitations of this study, the following conclusions were drawn:

1. Higher Pi and Fn growth levels may be anticipated when using the tested glass ionomer luting cement.
2. The combination of cement with titanium may exacerbate the bacterial growth capacity of Pi and Fn in contrast to their sole effect.

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