***Short- and long-term influence of fluoride-containing prophylactics on the growth of Streptococcus mutans on titanium surface***

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***ABSTRACT: (348 WORDS)***

***Objectives:*** Acidic pH and high fluoride($F^-$) concentration impair the corrosion resistance of titanium (Ti). $F^-$ may affect the oxide layer on Ti surface. Caries preventive mouthwashes and gels contain high amounts of $F^-$ and are applied at low pH. The purpose of the present study was to evaluate whether fluoride applied in different forms (like in the various caries prophylactic products) have different short-,mid- and long-term effects on the growth of the bacteria Streptococcus mutans (Str. mutans).

***Materials and methods:*** Commercially pure Ti grade 4 discs with a polished surface were treated with a rinse containing 0.025% fluoride, a gel containing 1.25% fluoride or a 1% aqueous solution of NaF (pH 4.5), and they were incubated with *Str. mutans* for 21 days. Control discs were incubated with the bacteria without prophylactic treatment. At the 5th, 10th and 21st days, the quantity of *Str. mutans* protein was measured by protein assay analysis. Scanning electron microscopic (SEM) images were also taken on the same days for qualitative analysis.

***Results:*** Although the SEM images showed difference between the treatments already on the 5th day of incubation, the difference in bacterial protein quantity became significant only by the 21st day. Significantly less bacterial protein was found on the surface of discs treated with fluoride in rinse and in gel, than on the surface of those treated with the aqueous solution of NaF ($p < 0.01$) and controls ($p<0.05$). Qualitative analysis revealed that while the gel and rinse treatment reduced the amount of bacterial protein content almost to the same extent, the gel treatment led to pitting corrosion on the Ti surfaces.

***Conclusion*:** The results suggest that the use of fluoride- containing mouthwashes might be the best and safest oral hygienic choice for patients with oral implants, while fluoride-containing gels may be just as safe to use even if in this specific study pitting corrosion was associated with the use of such a gel. The results also show that, when used for a longer period and at low pH, fluoride is more effective against biofilm development as olaflur than as NaF in aqueous solution.

***KEY WORDS:*** biofilm, fluoride, Streptococcus mutans, titanium, scanning electron microscopy (SEM)

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Titanium (Ti) and its alloys, due to their good biocompatibility, favorable mechanical properties and excellent corrosion resistance are widely used in dental and medical implants[1]. Surgical and endosseous dental implants for replacing or fastening hard tissue are made from commercially pure Ti (CP Ti)[2-3]. An insoluble titanium oxide ($TiO_2$) layer forms on the surface, which is why Ti and its alloys are resistant to corrosion. The oxide begins to form within nanoseconds in the presence of air and can reach a thickness between 20-100 Å in 1 s. It is impenetrable by oxygen and very adherent to Ti[4].

Oxidative processes are well known to thicken and condense the $TiO_2$ layer on the surface, protecting the metal from corrosion. On the contrary, reductive agents, such as fluorides, may have the opposite effect, that is, they may destroy this protective layer. Strietzel et al. demonstrated that in the presence of $F^-$, Ti ion release was increased and this reaction was even more pronounced at low pH[5]. Acidic pH and high ($F^-$) concentrations are described to weaken the corrosion resistance of Ti[6]. Crevice and pitting corrosion have both been described in such circumstances[7-8].

Patients frequently use various oral care products containing $F^-$, such as toothpastes, mouthwash solutions or other preventive gels. The scanning electron microscopy (SEM) research performed by Könönen et al., have observed that the use of topical $F^-$ containing agents causes stress corrosion on CP Ti[9].

Furthermore, such $F^-$ containing agents can come into contact with the transgingival part of Ti dental implants, exposed to the oral cavity. A rough surface promotes plaque accumulation on the peri-implant crevice, which is an undesired effect. Therefore, it is crucial to polish the rim of the neck part of implants[10-11] and the abutment, in order to prevent plaque accumulation. Without that, maximum bacterial colonization takes place in 24 hours on a pure Ti surface[12], and the bacterial count remains constant over a period of 14 days.

The study by Nakagawa et al. in 1999[13] proved that a high $F^-$ concentration at low pH causes the corrosion of CP Ti. The $TiO_2$ film is gradually destroyed in an acidic environment as a result of the reaction between

Ti and protons from the HF solution, even at low NaF concentrations[14]. A concentration of NaF above 0.1% has been described to eliminate the protective effect of the stable $TiO_2$ layer[15]. The pH of the rinses and gels used for caries prevention in dentistry are usually between 3.5 and 7.0, and the $F^-$ concentration is between 1,000 and 12,500 ppm[13]. Consequently, the question of the interaction between dental implants and fluoride-containing substances is one that must be dealt with. *Streptococcus mutans* is facultatively anaerobic, Gram-positive coccus commonly found in the human oral cavity and is known as a significant contributor to tooth decay[16-17].The dominant bacterial components of the dental plaque are the *Streptococcus* species (*Streptococcus sanguis and Streptococcus mutans*).

In an earlier study of ours[19] we dealt with how fluoride in gel, rinse and solution influences the growth of Streptococci on Ti surface. The incubation period in that study was 5 days, and no significant difference was found between the results yielded by the different treatments. We concluded, however, that such a short period does not allow drawing conclusions regarding the in vivo situation, where titanium implants are in the oral cavity for much longer, and therefore the really interesting question is the long-term effect of these prophylactic treatments. Because of that we decided to repeat the earlier study with a longer incubation period, and we hypothesized that a longer incubation period might bring different outcomes.

## MATERIALS AND METHODS

Mechanically polished Ti implant discs 9 mm in diameter and 2 mm in thickness (CP grade 4, Protetim, Hungary) were used. Similarly to the transgingival part of dental implants, the discs were polished to a surface roughness not exceeding 0,2 □m[10]. After cleaning in an ultrasonic bath with acetone, absolute ethanol and distilled water for 15 min, each sample was dried and immersed into a caries-preventive prophylactic. The applied prophylactics were a)mouthwash (Elmex, GABA International AG, Switzerland) containing 250 ppm $F^-$ (pH 4.4) in the form of Olaflur [*bis*-(hydroxyethyl)aminopropyl-*N*-(hydroxyethyl)octadecylamine dihydrofluoride] and potassium fluoride; b) aqueous solution of 1% NaF (3,800 ppm $F^-$, pH 4.5); c) gel (Elmex, GABA GmbH, Germany) containing a total of 12,500 ppm (1.25%)

$F^-$, pH 4.8, (2,500 ppm(0.25%) in the form of amine fluorides, Olaflur, Dectaflur (hexadecylaminehydrofluoride) and 10,000 ppm NaF). The pH of the NaF solution was reduced to 4.5 with lactic acid. The discs were always handled with Ti forceps so as not to contaminate the surfaces[18]. Each of the cleaned discs was used in only one experiment. For each experiment 48 Ti samples were used: 36 Ti discs were immersed into one of the prophylactic media mentioned above and 12 untreated (control)samples were not immersed, but went through the same cleaning procedure.

After an hour of immersion, the samples were removed from the $F^-$medium, washed completely with ultrapure water, and then dried. The application time corresponded to the accumulated effect of regular use of 4 months for the rinse, 7.5 months for the gel, assuming application according to the manufacturer's instructions. The solutions were always prepared fresh and filtered through a Millipore filter with 0.22 □m filter cartridge.

All of the treated and control discs were steam- sterilized at 160 °C for 45 minutes, in order to eliminate bacteria from the surfaces. Experiments were always performed within the validity time of the sterilization, which was 14 days[19]. The Ti discs were further divided into groups depending on the period of the incubation. The length of the incubation was 5, 10 and 21 days.

*Streptococcus mutans preparation*

Fresh colonies of the *Str. mutans* ATCC 25175 control strain, incubated in a 5% $CO_2$atmosphere for 24 h at 37 $^oC$, were suspended in reduced Brain Heart Infusion broth (pH 7,2; Oxoid, Basingstoke, United Kingdom) and used after gentle dispersion (McFarland 0.5 dilution). Two ml aliquots of this mixture were immediately plated onto 16-well sterile microtiter plates, containing the differently treated Ti discs. After 5,10 or 21 days of incubation under 5% $CO_2$, the samples were removed from the incubation.

*Protein assay studies*

The quantity of *Str. mutans* bacterial protein was determined with a micro BCA protein assay kit (Pierce, Rockford, IL USA), in order to check the survival and proliferation of the bacteria on the Ti discs surfaces were treated with the materials containing different amounts of $F^{-}$. To remove the bacteria, the samples were washed with a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$ and 1 μg/ml leupeptin).

*SEM studies*

After treatment with $F^{-}$and bacterial incubation, the Ti discs were treated with the following method for fixation: dehydration of the surface bacteria and bacterial biofilm, first by rinsing with ethanol solutions of increasing concentrations, (30–50–70–100%) then by a mixture of ethanol and acetone (90–10, 70–30, 50–50, 30–70, 10–90, 100% acetone). Critical point drying (an SPI 1320 apparatus) was applied, after which the discs were gold-coated by means of an Edwards sputter coater and subjected to SEM with a Hitachi S 2400 instrument.

*Statistical analysis*

Statistical analyses were conducted in Statistica for Windows (StatSoft, Inc., USA). Level of significance was set at ($p<0.05$).

**RESULTS**

Various amounts of biofilm evolved on the titanium disc surfaces depending upon the applied treatment. This is well illustrated by the Scanning Electron Microscope images taken at day 5. Figure 1 shows the surface of a disc treated with fluoride in rinse.

Figure 2 shows a SEM image of the surfaces of a Ti disc after treatment with fluoride in gel (12500 ppm $F^-$) at pH 4.8 and incubation with Str. mutans for 5 days. Corrosive regions and holes can be observed. Some bacteria formed multiple layers, but a definite biofilm is not present.

Figure 3 shows a remarkably rough Ti surface after a treatment with 1% NaF (3800 ppm $F^-$) at pH 4.5. After an incubation period for 5 days with Str. mutans a typical bacterial biofilm was formed in several layers on the surface.

After 5 days of incubation, the protein content measurement indicated no significant differences in the amount of bacteria on the surfaces of Ti discs after the different treatments (Fig.4).

Similarly to the 5-day results, the protein assays showed no difference between the differently treated groups of Ti discs, after an incubation period of 10 days (Fig.5).

However, a significant difference in the quantity of *Str mutans* could be observed after 21 days of incubation. A significant decrease in the amount of bacterial protein was observed in the groups treated with fluoride in rinse (250 ppm $F^-$at pH 4.4), or with fluoride in gel (12 500 ppm $F^-$at pH 4.8) as compared to the control and NaF groups (Fig. 6).

**DISCUSSION**

Our results confirmed our hypothesis that the different fluoride-containing prophylactics have a different influence on bacterial growth if a longer incubation time is allowed. To put it simply, our results suggest that in vivo the right choice of prophylactic does influence the resistance of Ti implant surfaces to bacterial growth, which we consider a clinically important finding.

After 5 days of bacterial incubation we could not observe significant quantitative difference between the bacterial protein content of the examined groups, however, the SEM images indicated difference already at this early point. At the 10th day of incubation, the difference was still only slight, but the superiority of fluoride in rinse and gel was becoming evident. It must be mentioned that the gel-treated surfaces showed pitting corrosion, which the rinse-treated surfaces were free of. By the twenty-first day of incubation fluoride in rinse and gel proved to be unambiguously superior to the aqueous solution of NaF and no treatment. At this time point significantly less bacterial protein was detected on the surface of discs treated with fluoride in rinse and gel as compared to NaF-treated discs and controls, which was supported also by the SEM images. Considering both the quantitative (protein assay) and qualitative (SEM) results of this study, the most effective treatment was fluoride in rinse. Although the efficiency of fluoride in gel and fluoride in rinse was comparable, the rinse form did not damage the surface of the polished Ti discs, which made it superior to the gel form, as the pitting corrosion caused by the latter favored bacterial colonization.

Which form of fluoride delivery is the optimal for use with Ti for prophylactic purposes was not a question of this study, but the results suggest that this is indeed a significant factor. According to what we found, fluoride (olaflur) in rinse yields the best results, while the aqueous solution of sodium-fluoride appears to be ineffective. Not having done any specific tests that could allow us to explain these findings, we would like to avoid speculations here- however, why the gel format causes corrosion and why sodium fluoride in aqueous solution seems to be completely ineffective would certainly deserve further research. At the same time, the finding about the gel form and corrosion should not be interpreted as an absolute contraindication of this vehicle. It has to be kept in mind that fluoride in gel and rinse yielded nearly the same results, which means that clinically they are likely to be similarly efficient and safe. Also, it might be that corrosion occurs only with the specific type of prophylactic gel we applied, and that it is not characteristic of gel as a vehicle in general.

**CONCLUSION**

These results suggest that the use of fluoride- containing mouthwashes is the best and safest oral hygienic choice for patients with oral implants, while fluoride-containing gels may be just as safe to use even if in this specific study pitting corrosion was associated with the use of such a gel. Our study also suggests that, when used for a longer period and at low pH, fluoride is more effective against biofilm development as olaflur than as NaF.

**DISCLOSURE**

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**LEGENDS**

**Fig.1.** SEM image of bacteria on a Ti disc. The disc was previously treated with fluoride in rinse (250 ppm $F^-$ ) at pH 4.4 and then incubated with *Str. mutans* for 5 days(Magnification:8 000×).

**Fig. 2.** SEM image of a Ti disc treated with fluoride in gel (12500 ppm $F^-$) at pH 4.8 and incubated with Str. mutans for 5 days. Pitting corrosion (holes) and bacteria can be detected on the surface.(Magnification: 5 000×).

**Fig. 3.** SEM image of bacteria on Ti discs treated with 1% NaF (3 800 ppm $F^-$) at pH 4.5 and then incubated with Str. mutans for 5 days. Biofilm development is obvious. (Magnification: 6 000×).

**Fig 4.** Bacterial protein content on the surface of Ti discs after 5 days of incubation with *Str. mutans*.

**Fig. 5.** Protein amounts measured after 10 days of incubation with *Str. mutans*.

**Fig 6.** Bacterial protein content at day 21. Fluoride in rinse (57.62 µg/ml ±8.96) and fluoride in gel (59.99 µg/ml ±7.58) yielded similar results, but both turned out to be significantly more efficient than NaF in solution (83.42 µg/ml ±14.36; $p<0.01$) and no treatment (81.54 µg/ml ±17.37; $p<0.05$). It is to be noted that the efficiency of NaF in solution was so low that this treatment yielded almost exactly the same results as no treatment.