Electronic Physician (ISSN: 2008-5842) http://www.ephysician.ir
March 2017, Volume: 9, Issue: 3, Pages: 4042-4047, DOI: http://dx.doi.org/10.19082/4042

Comparison of antimicrobial effects of titanium tetrafluoride, chlorhexidine, xylitol and sodium fluoride on *streptococcus mutans*: An in-vitro study

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Type of article: Original

Abstract

Introduction: No studies have yet documented the bactericidal effects of TiF4, and its role in the treatment of dental caries, and no definite protocol has been introduced to regulate its use. The aim of this study was to determine the antimicrobial/bactericidal effects of TiF4 on *Streptococcus Mutans* (*S. Mutans*) and to compare it with chlorhexidine (Chx), sodium fluoride (NaF) and xylitol.

Methods: This study was conducted at the Shiraz University of Medical Sciences microbiology laboratory during March 2015 to September 2015. In this in-vitro study, first a bacterial suspension was prepared and adjusted to a 0.5 McFarland standard (equivalent to 1×10^8 CFU/ml). The minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) of TiF4, Chx, NaF and xylitol were assessed using broth microdilution assay and disk diffusion methods. In order to neutralize the acidic nature of TiF4, we used a sodium hydroxide preparation to obtain a pH of 7.2 and repeated all of the previous tests with the neutralized TiF4 solution. We reported the final results as percentages where appropriate.

Results: The MIC of TiF4, NaF and Chx for *S. Mutans* were 12.5%, 12.5% and 6.25%, respectively. At a concentration of 12.5% the inhibition zone diameters were 9 mm, 15mm and 14mm for TiF4, NaF and Chx, respectively. The MBC was 25%, 12.5% and 12.5% for TiF4, NaF and Chx, respectively. Xylitol failed to show any bactericidal or growth inhibitory effect in all of its concentrations. When we repeated the tests with an adjusted pH, identical results were obtained.

Conclusion: TiF4 solutions have anti-growth and bactericidal effects on *S. Mutans* at a concentration of 12.5% which is comparable with chlorhexidine and NaF, indicating the possible use of this solution in dental practice as an anti-cariogenic agent, furthermore the antimicrobial activity is unaffected by pH of the environment.

Keywords: Antimicrobial; Titanium tetrafluoride; Chlorhexidine; Sodium fluoride; Xylitol; Caries

1. Introduction

Dental caries are the result of complex interactions between various host, substrate, agent and time related factors (1). Aside from the genetic and environmental factors that predispose a person to develop caries, the process itself

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Received: June 12, 2016, Accepted: July 12, 2016, Published: March 2017
iThenticate screening: July 10, 2016, English editing: September 20, 2016, Quality control: December 14, 2016

does not occur without the existence of dental plaque or fermentable carbohydrates. Multiple studies have documented an association between an increase in number of *S. Mutans* in the saliva, dental plaque and increase in dental caries (2). Nowadays the serotypes of *S. Mutans* are considered the primary microorganism involved in caries formation, therefore correct control of *S. Mutans* levels is among the most important goals in the prevention and treatment of caries (3). Among the treatment modalities which have been introduced throughout the years, chemotherapeutic regimens have displayed promising results and have been a topic of interest in recent studies. These agents have been considered for their ability to reduce acid formation in dental plaque (resulting from special types of bacteria like *S. Mutans*). Fluorides are among these agents which have emerged as the leading preventive substance for their ability to reduce caries formation. Treatment with fluoride has been recommended for children with moderate to high risk of caries formation, by the American Academy of Pediatric Dentistry (4). In clinical practice, various antimicrobial compounds and solutions have been studied for their role in the prevention and eradication of oral caries aside from the more commonly used fluorides. These include: chlorhexidine (Chx) which is considered the "gold standard" of oral antiseptics and is the most studied anti-cariogenic agent up to this date (5) and xylitol which is normally present in fruits and has been documented to have anti-cariogenic properties (6). Recently titanium tetraflouride (TiF4) has been considered for its ability in preventing enamel demineralization (7, 8). TiF4 was first introduced in 1972 and although studies have demonstrated its benefits as an anti-erosive agent, a definite protocol to regulate its use has not yet been introduced, which makes it difficult to obtain in the local market (9). TiF4 solution seems to have an inhibiting effect on caries formation (10), however no studies have yet documented the bactericidal effects of TiF4 and its role in the treatment of dental caries (11). When compared to other topically used fluorides like sodium fluoride, which is commonly used for dental caries prevention (9, 12), TiF4 seems to render better results, with higher penetration and uptake of fluoride and lower acid solubility of tissues (13). The aim of this study was to evaluate the antimicrobial effects of TiF4 on *S. Mutans* and to compare it with Chx, NaF and xylitol.

2. Material and Methods

2.1. Study protocol

The study protocol was approved by the institutional review board of Shiraz University of Medical Sciences and was conducted at the Shiraz University of Medical Sciences microbiology laboratory during March 2015 to September 2015. TiF4 (Sigma, USA), xylitol (Sigma, USA), chlorhexidine (Behsa, Iran) and NaF (Behsa, Iran) 0.2% mouth rinse were obtained. Lyophilized *Streptococcus Mutans* ATCC 35668 was purchased from the Persian Type Culture Collection (PTCC), Iran.

2.2. Inoculation of culture media with reference strain

The lyophilized *S. Mutans* was first added to a triptycase soy broth media. The media was then incubated for 24 hours (H2:CO2:N2=10:10:80 and at a temperature of 37 °C). The cultivated bacteria were then streaked onto a blood agar media and were incubated for another 24 hours. A well isolated colony was selected from the agar plate and was transferred into a sterile 4ml nutrient broth medium in a sterile manner. The final broth medium was then incubated for 24 hours (H2:CO2:N2=10:10:80 and at a temperature of 37 °C). The turbidity of the bacterial suspension was adjusted to a 0.5 McFarland standard (which is equivalent to a 1×10^8 CFU/ml bacterial suspension) for the related antimicrobial susceptibility testing.

2.3. Broth microdilution assay

In order to obtain the broth microdilution susceptibility, a panel which included 96 wells was prepared. First 100 µl of nutrient broth was inserted into all of the wells (except for the last two wells). For obtaining susceptibility concentration, 100 µl of TiF4 was inserted into the first well, after which, to acquire a two-fold serial dilution for TiF4, 100 µl of solution was extracted from the first well and inserted into the second well. This was repeated until reaching a concentration of 3.125%. The process was repeated for all of the agents in a separate panel. After preparing the concentration levels for each of the agents, a 0.5 McFarland bacterial suspension was diluted with a ratio of 1:10 to yield 10^7 CFU/ml. Five microliters of the bacterial suspension was then inoculated into each of the wells, obtaining a final bacterial concentration of 5×10^4 CFU/well. In each of the plates the last remaining two wells were considered as positive and negative controls. Positive control was a well that included only a sole inoculation of bacteria and negative control was a well that did not have any bacterial inoculation. The 96-micro well plates were then covered with a perforated plate seal and incubated for 48 hours (H2:CO2:N2=10:10:80 and at a temperature of 37 °C). Minimal inhibitory concentration (MIC) was considered as the lowest concentration where no viability of microorganism was detected after the incubation period. Each test was repeated three times for minimizing lab related errors. In order to obtain the minimal bactericidal concentration (MBC) for each agent, 10µl

of broth medium was collected from those wells that had not shown any signs of growth (at different concentrations) and was inoculated on to a blood agar medium. To obtain a control group, another blood agar medium was inoculated with the *S. Mutans* bacteria. After 24 hours of incubation (37 °C), the lowest concentration at which no growth had occurred, was considered as the MBC. For Chx, xylitol and NaF the same steps were followed accordingly.

2.4. Disk diffusion method

A sterile disk was first impregnated with 30µl of undiluted TiF4, xylitol, NaF and Chx. After the disks had dried, each disk was transferred to an inoculated blood agar medium, using sterile forceps. The plates were then incubated for 24 hours (H2:CO2:N2=10:10:80 and at a temperature of 37 °C) and the inhibition zone diameter was measured using a ruler. Each test was repeated three times to minimize lab errors. One disk which was soaked in distilled water was placed on each plate as a negative control. To better evaluate the antimicrobial effects of TiF4 and to minimize the effects of low pH (which is provided by the TiF4 solution) on *S. Mutans* growth, we first calculated the pH provided by the TiF4 solution which was 5.8. After which, in order to neutralize the acidic effect of the TiF4 solution, we used a sodium hydroxide (NaOH 1N, Merck, Germany) preparation to obtain a pH of 7.2, and repeated all of the previous tests with the neutralized TiF4 solution.

2.5. Statistical analysis

Since we did not record any deviation in our measurements, we reported the final results as percentages where appropriate.

3. Results

When determining the MIC of TiF4, NaF, Chx and xylitol using the broth microdilution assay, we found that the MIC of TiF4 solution for *S. Mutans* was similar to that of NaF (12.5%). Chx had the lowest MIC (6.25%) in comparison to TiF4 and NaF. At a concentration of 12.5%, the inhibition zone diameters were nine mm, fifteen mm and fourteen mm for TiF4 NaF and Chx, respectively. TiF4 failed to show any inhibitory effect at a lower concentration of 6.25%. Xylitol did not show any inhibitory effect in any of its concentrations (Table 1). Evaluating the MBC using the disk diffusion method, we found that the MBC of NaF for *S. Mutans* was similar to that of Chx (12.5%), meanwhile TiF4 solution had a higher MBC (25%). As expected xylitol failed to show any bactericidal activity in all of its concentrations (Table 2). When we repeated the broth microdilution assay and the disk diffusion method for evaluating the MIC and MBC with an adjusted pH (pH=7.2), the exact same results were obtained.

Table 1. Inhibitory effect of each agent at different concentrations and their related inhibition zone diameters.*

Agents	Inhibitory concentration – Inhibition zone diameter (mm)				
	3.125%	6.25%	12.5%	25%	50%
TiF4	N	N	Y – 9	Y – 15	Y – 22
NaF	N	N	Y – 15	Y – 17	Y – 22
Chx	N	Y – 1	Y – 14	Y – 19	Y – 21
Xylitol	N	N	N	N	N

TiF4: Titanium tetra-fluoride; NaF: sodium fluoride; Chx: chlorhexidine; Y: yes; N: no; * The minimal inhibitory concentration was 6.25%, 12.5% and 12.5% for Chx, NaF and TiF4, respectively.

Table 2. Bactericidal effect of each agent at different concentrations.*

Agents	Bactericidal solution concentration				
	3.125%	6.25%	12.5%	25%	50%
TiF4	N	N	N	Y	Y
NaF	N	N	Y	Y	Y
Chx	N	N	Y	Y	Y
Xylitol	N	N	N	N	N

TiF4: Titanium tetra-fluoride; NaF: sodium fluoride; Chx: chlorhexidine; Y: yes; N: no; * The minimal bactericidal concentration was 12.5%, 12.5% and 25% for Chx, NaF and TiF4, respectively.

4. Discussion

Here we evaluated the antimicrobial effects of TiF4 and compared it to NaF, Chx and xylitol which are the more commonly used antimicrobial agents for the prevention and treatment of oral caries (associated with *S. Mutans*). We

found that, similar to NaF, the minimum inhibitory effect of TiF4 starts at a concentration of 12.5%. The bactericidal and growth inhibiting effects of TiF4 was maintained, even when the pH of the solution was adjusted to a neutral pH. To the best of the authors' knowledge this study is among the first to evaluate the antimicrobial/bactericidal effects of TiF4 solution against other more common antimicrobial solutions for *S. Mutans*. Our results showed that TiF4 had remarkable antimicrobial activity and this is probably due to both the fluoride substance and the titanium rich covered layer which is formed on the enamel surface exposed to it, which in return reduces its solubility and prevents its cariogenic threat (14). In recent years, TiF4 has been primarily studied for its anti-erosive effects. Among these studies, Souza et al. (15) evaluated an experimental solution of TiF4 and NaF in 2014. They documented that a solution containing a 0.0815% concentration of TiF4 had the highest anti-erosive effect with a 99% reduction in enamel wear followed by a solution containing 0.042% NaF and 0.049% TiF4, which had a 41% reduction in enamel wear. They concluded that a pure solution of TiF4 had the strongest anti-erosive effect. This finding was also seen in the study by Catilho et al. (16) in which they documented a solution containing 0.0815% TiF4 to have the strongest effect in reduction of dentin loss. Similar findings were also seen in other studies evaluating the role of TiF4 as an anti-erosive agent (17-20). Studies on the effects of TiF4 as a preventive agent for caries formation have been few and mostly old. One review published in 2010 by Wiegand et al. evaluated the role of TiF4 as a preventive agent for the development and progression of carious and erosive lesions. They reported that based on previous in-vitro research, TiF4 is effective in preventing carious lesions and is equally or more effective than NaF, amine fluoride or stannous fluoride (SnF2). The study concluded that TiF4 is a considerable agent for the prevention of caries formation (21); although, that review mostly included old studies evaluating the topical application of TiF4 on caries prevention. In another review in 2011 by Wahengbam et al., (9) the preventive role of TiF4 in dentistry was studied, and as part of their investigation the cariostatic effects of TiF4 was also investigated. They attributed the protective effects of TiF4 preparations to two factors: first to its chemical effects in decreasing enamel solubility by increasing fluoride content and secondly, providing a glaze on the surface of the teeth, which is resistant to acid penetration. They concluded TiF4 to be more effective than other metal based fluorides in the prevention of caries. In a study in 2015 by Bridi et al. (22), the antimicrobial effects of TiF4 solution (2.5%) followed by self-etching adhesives was evaluated on *S. Mutans.* After applying the solution on 40 molar teeth cavities, they documented the solution to have antimicrobial effects on *S. Mutans*. In a past study by Reed and colleagues, they evaluated the effects of topical application of TiF4 on caries formation. They found that TiF4 with a concentration of one percent was more effective in caries prevention, when compared to the equivalent solution (in wise of concentration) of SnF2, NaF and aluminum phosphate fluoride. This finding was also supported by much earlier studies (23, 24). Although the xylitol solution did not display any antimicrobial effects in our study, some previous studies have documented it to have inhibiting effects on bacterial growth. Milgrom et al. (25) evaluated three groups of people: those not using xylitol and those using xylitol containing gums at a level of 6.88g/day and 10.32 g/day. They found that those patients who chewed xylitol containing gum, had a decrease in their oral number of *S. Mutans.* Up to this day no study has reported xylitol to have any cariostatic effect on *S. Mutans.* Our study also did not show xylitol to have any bactericidal effects on *S. Mutans*, although our results might have been effected by the pH of the environment and the material which we used in the study. Some points have to be considered regarding TiF4 solutions. First is the acidic nature of TiF4 which should be considered, although titanium lacks irrigating properties and in itself is non-toxic (9). The high fluoride levels in TiF4 solutions may have the risk of overdose and possible damage to the teeth, so caution must be taken when considering TiF4 for clinical application. Our study had an in-vitro design which limits its application in clinical practice and further studies with possible human subjects should be considered in the future.

5. Conclusions

The results of our study showed that TiF4 solutions do have anti-growth and bactericidal effects on *S. Mutans*, which is comparable with chlorhexidine and NaF, indicating the possible use of this solution in dental practice as an anti-cariogenic agent for the treatment of caries, furthermore the antimicrobial effect is not influenced by the pH of the environment. Perhaps pilot studies with human subjects in controlled conditions would aid in better evaluating the clinical perspectives of TiF4 solutions.

Acknowledgments:

The authors would like to thank the vice-chancellery for research at Shiraz University of Medical Sciences for their support of this study (Grant #94940). The manuscript is part of the thesis of Dr. Zahra Abdoli. The authors would also like to thank Mohsen Hosseinifarzad for his assistance in the laboratory.

Conflict of Interest:
There is no conflict of interest to be declared.

Authors' contributions:
All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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