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Original Article

An In vitro Analysis of the Effects of Iron Sulfate and Iron Acetate on *Streptococcus mutans*

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Abstract

Statement of problem: Dental caries is a common infectious disease induced by *Streptococcus mutans (S. mutans)*.

Objectives: Due to the high incidence rate of dental caries and iron deficiency in the Iranian population, we have conducted this study to analyze the effects of iron acetate and iron sulfate on controlling the growth of *S. mutans*.

Materials and Methods: In this in vitro study, we evaluated the antibacterial effects of iron sulfate and iron acetate on *S. mutans* by the disk diffusion method, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC). The results were compared to those for 0.2% chlorhexidine and penicillin as the controls.

Results: Iron sulfate had higher MIC and MBC values compared to penicillin and chlorhexidine ($P<0.001$). Iron acetate MIC and MBC values did not significantly differ with penicillin and chlorhexidine. The iron sulfate inhibition zones at the 25 and 50 µg/mL doses were more than those of iron acetate.

Conclusions: Iron sulfate and iron acetate solutions can inhibit the growth of *S. mutans*. Hence, different compounds that contain iron salts such as toothpastes, mouth washes, and food supplements can be produced to prevent dental caries and iron deficiency.

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Introduction

Dental caries is a common infectious disease that results from mineralized tissue dissolution by cariogenic bacteria. *Streptococcus mutans (S. mutans)* is a common cariogenic bacterium [1]. This gram positive, non-mobile, anaerobic bacterium can produce acid and ferment food remnants, which result in teeth structure demineralization [1]. This bacterium attaches itself to the tooth's surface with its outer cell polysaccharide [2,3]. The iron concentration in people's saliva depends on their diet and physiologic lactoferrin levels. According to previous studies, iron appears to affect the aggregation and attachment of bacteria to the tooth's surface; hence, iron can impact the development or prevention of dental caries [4-7]. Dunning *et al*. discovered the anti-caries effects of the iron ion [8]. In 2001, Devulaplle *et al*. concluded that iron ions inhibit glycosyltransferase enzyme (GTF) produced by *S. mutans* [9].There are different methods to prevent dental caries such as oral hygiene, dental floss, fluoride gels, fissure sealants, and mouthwashes [10]. In recent years, evaluation of antibacterial effects of different ions has been proposed as new complimentary and substitute methods to prevent caries. This study aims to analyze iron acetate and iron sulfate on *S. mutans* growth in vitro.

Materials and Methods

We prepared a standard strain of *S. mutans* (ATCC 35668, PTCC 1683), obtained from the Iranian Organization for Science and Technology, Tehran, Iran.We prepared iron sulfate (Sigma Chemical Co.) and iron acetate (Sigma Chemical Co.) solutions at concentrations of 6.25, 12.5, 25, and 50 µg/mL. The prepared concentrations were sterilized by autoclave. In order to determine the antimicrobial effect of iron sulfate and iron acetate on *S. mutans*, we used the disk diffusion technique [11]. A bacterial suspension of *S. mutans* was adjusted to 0.5 McFarland turbidity (1.5x108cfu/mL) in normal saline, after which it was cultured in blood agar for 24 hours. The bacterial suspension was applied with a sterile cotton swab on Muller Hinton agar (MHA, Merck, Germany) with 5% sheep blood. Then, 20 µL of either the iron sulfate concentration or iron acetate were applied to sterile, air-dried paper disks (Padtan Teb, Iran). The positive controls were 10 unit penicillin disks (Mast, UK) and dried plain paper disks that contained 20 µL of 0.2% chlorhexidine. We incubated the plates at 37°C for 24 hours and subsequently measured the inhibition zones in millimeters.The broth dilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of iron sulfate and iron acetate. We added 100 µL of brain heart infusion broth (BHI, Hi-Media, India) to each well of 96-well microtiter plates. Then, 100 µL of the stock solutions were added to the wells in sequential order (final concentrations: 4.8-250 µg/mL). The well that only contained BHI medium was the negative control. The positive control wells were penicillin and 0.2% chlorhexidine, and a well that contained BHI and bacterial suspension. The microwell plates were incubated at 37°C in a 5% CO2 incubator for 24 hours. The first well that inhibited bacterial growth was considered to be the MIC. We also cultured 10 µL of the well contents without any bacterial growth on blood agar to evaluate the MBC. The plates were incubated for 24 hours at 37°C in a 5% CO2 incubator. The least concentration that did not show any *S. mutans* colony formation on the agar was considered to be the MBC. These evaluations were rendered twice with a one-week interval. SPSSTM software, version 18.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Non-parametric Kruskal-Wallis and Mann Whitney tests were used to compare different groups. $P<0.05$ was considered statistically significant.

Results

We used the disk diffusion method to determine the inhibitory ability of the studied solution on *S. mutans* growth. There was no inhibition zone observed at the 6.25 µg/mL concentration of iron sulfate and at 12.5 µg/mL of iron acetate. At 25 µg/mL of iron sulfate, the inhibition zone (7.07±5 mm) did not statistically differ from the inhibition zone for iron acetate (7.87±5.5 mm; $P>0.05$). However, the inhibition zone for 50 µg/mL iron sulfate (15±4.24 mm) was more than the inhibition zone for iron acetate (11.31±8 mm). This finding showed a more potent inhibitory effect of iron sulfate ($P<0.05$) at that concentration. The 0.2% chlorhexidine solution (control) had an inhibitory zone of 16.5 mm, which was significantly

higher than the iron sulfate and iron acetate groups (P<0.05). Table 1 shows the mean MIC and MBC of the studied salt solutions in comparison with penicillin and 0.2% chlorhexidine. We observed a significantly greater MIC of iron sulfate (P<0.001), which indicated that iron sulfate had less antibacterial properties compared to iron acetate, penicillin, and 0.2% chlorhexidine. This finding showed the anti-*S. mutans* activity of this salt relative to penicillin and 0.2% chlorhexidine. The 0.2% chlorhexidine had a statistically higher MIC than penicillin (P=0.009). Iron sulfate had a significantly higher MBC compared to the other groups (P<0.001). According to the MIC and MBC results, iron sulfate showed the lowest inhibitory effect. Iron acetate had a more comparable inhibitory effect on *S. mutans*. Penicillin MBC was significantly higher compared to 0.2% chlorhexidine (P=0.009; Table 1).

Table 1: Mean MIC and MBC of the studied salt solutions in comparison with penicillin and chlorhexidine

Groups	MIC (µg/mL)	MBC (µg/mL)
Iron sulfate	586	1172
Iron acetate	108	234.4
Penicillin	1.3	2.7
Chlorhexidine	0.95	1.95

MIC= Minimum inhibitory concentration
MBC= Minimum bactericidal concentration

Discussion

In this study, we evaluated the antibacterial effects of different concentrations of iron sulfate and iron acetate. Iron sulfate inhibited *S. mutans* at higher concentrations compared to iron acetate. However, the inhibition zones for iron sulfate and iron acetate solutions were approximately the same. These salts have shown no significant differences in comparison with 0.2% chlorhexidine and penicillin, as the controls. In the disc diffusion method, one of the effective factors for production of an inhibitory zone is diffusion of salt solutions in the agar. Hence, it seems that the decreased solubility of iron acetate in saline and lack of its proper diffusion in agar could be the reason for the smaller zone of inhibition. The findings of this study were similar to previous studies that reported the inhibitory effect of iron salts on *S. mutans* growth. However, those studies were conducted under different conditions and different concentrations. Berlutti *et al*. reported that a concentration of Fe3+ lower than 0.1 µM caused aggregation and proliferation of *S. mutans*. Concentrations higher than 1 µM showed no effect [6]. These findings confirmed our results with Fe2+; however, Berlutti *et al*. have tested the effects of Fe3+ salts on bovine teeth. We believe that the results of this study might be more accurate. Unlike Fe3+, Fe2+ is not harmful for humans and does not change to Fe3+. In addition, vitamin C can be prescribed along with Fe2+ion [12]. A study conducted by Dunnin *et al*. has shown that 10 mM of Fe2+ was more effective than Fe3+ and had bactericidal effects at a pH of 7-8 [8].Our results supported the findings of an in vitro study conducted by Al Shalan *et al*., in which they observed the effect of 4 iron supplements on dental carries. The process of demineralization and cavity induction on teeth was observed during 60 days under in vitro conditions. With the exception of ferrous, all other supplements that contained iron ion had significant inhibitory effects on cariogenesis [13]. A study by Alves KM. *et al* in 2011 reported that ferrous sulfate decreased demineralization, but did not allow re-mineralization [14]. Eskandari *et al*. also confirmed that ferrous sulfate supplements could not cause enamel demineralization [15]. Another study revealed that an iron solution could decrease enamel surface microhardness and wear in the dentin; hence, loss of dental structure could be controlled by rinsing the mouth with the iron solution, especially after erosive trauma [16]. Pecharki *et al*. showed that iron ion reduced the sucrose potential of cariogenesis by decreasing the population of *S. mutans* in dental biofilm [17]. Ribeiro *et al*. in a study on the anticariogenesis mechanism of iron ion, reached the conclusion that iron, like chlorhexidine and sodium fluoride, decreased demineralization of the enamel. This effect was achieved by reducing the bacterial population rather than inhibition of glucosyltransferase enzyme [18]. In order to eliminate the confounding factors, the present experiment was repeated 3 times with

2 different iron salts. These studies evaluated the effects of iron ion on demineralization, cariogenesis, enamel, and dentin surface microhardness. We could not compare those results with the details of the present study methodology. The previous studies reported inhibition of cariogenesis by the effects of different aspects of the cariogenesis processes.There are several prescriptions to reduce the oral microbial count that have both positive points and adverse effects. Chlorhexidine mouthwash has a wide range of antibacterial properties. It is prescribed to decrease the level of oral bacteria, especially before and after oral surgery, and for periodontal diseases. The adverse effects of this product, as with other oral hygiene products, include teeth discoloration and dental stain accumulation. It can also cause severe hypersensitivity reactions. If swallowed, chlorhexidine can cause stomach inflammation, bradycardia, cyanosis, and ultimately liver damage. Chlorhexidine gluconate is not prescribed for children under the age of 18. This product can affect taste for up to 4 hours after use [19-21]. On the other hand, the use of antibiotics for an extended period can cause antibacterial resistance; thus, it seems to be of benefit to investigate new antimicrobial agents [2,3]. In the present study, we did not have access to pure chlorhexidine powder. Thus, we used the mouthwash as a replacement, which could justify its lower MIC and MBC in comparison to chlorhexidine. Another limitation was the use of citric acid as the solvent due to the low solubility of iron acetate in water. The use of this acid might affect the antibacterial effects of this salt. The authors suggest that additional experiments should be conducted on other, more water soluble iron salts, or in combination with other salts that do not have antagonistic effects at different concentrations, or on other cariogenic bacteria. Finally, this study should be conducted as an in vivo experiment.

Conclusions

In this in vitro study, iron sulfate and iron acetate inhibited the growth of *S. mutans* as a dental cariogenic bacterium. Although the antibacterial effects of the iron salts were lower than chlorhexidine, we suggest an in vivo study to determine the effects of these solutions. Several products that contain iron salts, such as toothpastes, mouthwashes, and food supplements can be developed in order to take advantage of the antibacterial properties of the iron salts.

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Conflict of Interest: None declared.

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