

***In vitro* effect of different concentrations of iron on the initiation of dental caries: Pilot study**

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أوضحت الدراسات الحيوانية السابقة أن الحديد يقلل من حدوث النخور السنية. هدفت هذه الدراسة المخبرية إلى الكشف عن تأثير التراكيز المختلفة لعنصر الحديد على بدء النخور السنية. شملت الدراسة ستون (٦٠) ضاحكه سليمة مخلوغة قسمت عشوائياً إلى خمس مجموعات (١٢/مجموعه). المجموعه التحكمية الموجبة (مجموعه ١) لم يتم إضافة الحديد إليها. المجموعات التجريبية تكونت بإضافة الحديد بتركيز ١٠٠٪ و ٥٠٪ و ٢٥٪ و ١٢,٥٪ (المجموعه ٢ و ٣ و ٤ و ٥ على التوالي). تم إستعمال المكورات العقدية المتحول (٦٧١٥) النامي في الوسط. بعد ذلك وضعت الأسنان في صفائح حاوية على الوسط المكون من الحديد والجراثيم والسكروز وتم تغيير الوسط يومياً لمدة ٢٩ يوماً. تمت مراقبة العينات يومياً بشكل عياني لتحري وجود تجاويف أو أي مناطق منزوعة الكلس. دلت النتائج على أن كافة المجموعات التي تلقت الحديد أبدت إنخفاضاً في نسبة المناطق المنزوعة الكلس والأفات نخرية مقارنة مع المجموعه الموجبه. لم يحدث نخور سنية في جميع المجموعات التجريبية طوال فترة الدراسة (٢٩ يوم). يمكن من خلال ذلك إستنتاج أنه قد يكون للحديد فعالية مضادة للنخر في أسنان الإنسان.

Previous animal studies have shown that iron reduced the incidence of dental caries. **Objective:** The purpose of this *in vitro* study was to examine the effect of different concentrations of iron-supplement on the initiation of dental caries on human teeth. **Materials and Methods:** Sixty extracted human premolar teeth were randomly distributed into five groups (12/group). Positive control group (Group 1) did not receive any iron. Experimental groups consisted of adding iron in 100%, 50%, 25% and 12.5% concentrations as Groups 2,3, 4, and 5 respectively. Mutans Streptococci (MS) bacteria (6715) grown in Todd Hewitt Broth were used. The teeth were placed in 24-well ELISA plates (two groups / plate). On each well, a media consisting of iron (for experimental groups), bacteria and 10% sucrose was added and changed daily for 29 days. Daily assessment of any decalcification and cavitation was done. **Results:** All groups receiving iron developed significantly fewer decalcification and carious lesions compared with the positive control. In addition, no cavitation was seen in all the experimental groups during the study period. **Conclusion:** It is concluded that iron may have some protective action against the development of dental caries in human teeth.

INTRODUCTION

Dental caries remains the most infectious disease affecting humans. It is caused by bacteria that are harbored in dental plaque. Mutans streptococci (MS) have been identified as the principal bacteria causing dental caries in humans.¹ MS bacteria, under specific conditions, can ferment sugars and other carbohydrates from foods and drinks to produce lactic acid and other short chain organic acids.² If the concentration of the acid depresses the pH adjacent to the tooth surface below 5.5, then the enamel may dissolve. Glucosyltransferase (GTF) enzymes from MS play a pivotal role in dental caries

and are considered the most significant virulence factor.¹

Different methods of caries prevention are in the dental literature.³ Previous studies had investigated the effect of minerals on the inhibition of dental caries.⁴⁻⁸ The results of previous experiments suggested that iron (Fe) added to a cariogenic diet could reduce the incidence of dental caries in animals.^{9,10} In addition, Rosalen *et al.*¹¹ and Miguel *et al.*¹² have found that iron decreases the caries development in de-salivated rats. Moreover, Larsson *et al.*¹³ found that diet supplemented with iron salts, either in food or in drinking water may have cariostatic effect attributable primarily to local action on the teeth.

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Other studies have also shown that Fe ions have a strong inhibitory effect on the GTF enzyme. This may explain the inhibitor effect of iron.¹⁴

No previous studies have investigated the effect of different concentrations of iron on the initiation of dental caries on human teeth using controlled environment such as artificial caries study. Therefore, the purpose of this *in vitro* study was to determine the effect of different concentrations of iron on the initiation of dental caries on human teeth.

MATERIAL AND METHODS

Bacteria Strain

MS bacterial strain (6715) was used in this study. The stock organism was stored in skim milk at -80° C until use. Cultures of these MS strain were initiated from frozen stocks by incubating five loops of the bacteria stock in 500 ml Todd-Hewitt broth (DIFCO, Detroit, MI) grown anaerobically in brewer jar filled with 80% N₂, 10% H₂, and 10% Co₂ at 37°C.

Teeth Preparation

Sixty extracted caries-free and restoration-free human premolars were used. These teeth were collected, cleaned and stored using thymol at room temperature until used. Teeth were mounted using cold cure acrylic resin, which covered the tooth up to the cemento-enamel-junction. Selected area on the buccal surface of the teeth were covered with a drop of nail polish (colored) then the whole coronal part was covered with transparent nail polish (two layers). After the varnish dried, the colored part was removed to leave one exposed enamel surface of approximately 0.5 cm². The teeth were randomly divided into five groups (Table 1).

Table1. Description of the groups and the material used

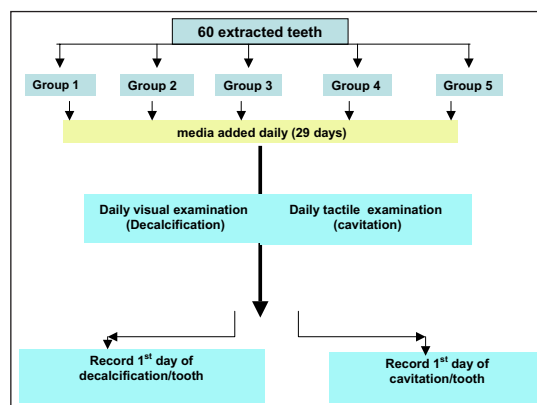
Group	Name	Media		
		Bacteria	Sucrose	Iron
1	Positive control	1.00 ml	200 ul	00*
2	100% Fe	1.00 ml	200 ul	200 ul
3	50% Fe	1.00 ml	200 ul	200 ul
4	25% Fe	1.00 ml	200 ul	200 ul
5	12.5% Fe	1.00 ml	200 ul	200 ul

* 200 ul dH₂o was used instead of iron.

Artificial Caries Experiment

The experimental design is shown in Figure 1. Three 24-well ELISA plates (2ml volume/well) were used. The mounted teeth were immersed in solution media containing MS bacteria strain (1x10⁷ cells), 200ul 10% sucrose and 200 ul Fe in different concentrations (Table1). The teeth in solution were incubated in anaerobic chamber at 37°C for 24 hours. Fresh sucrose, iron and bacteria were added daily for four weeks.

Figure 1. Flowchart of the experimental design of the study.



Caries Progress Evaluation

The progress of dental caries was randomly evaluated by daily visual examination for decalcification and by

tactile examination for cavitation using the explorer. The dates and degree of enamel decalcification and caries progression were recorded daily.

Statistical Analysis

Data were entered using FOXPRO data base program. Differences in caries initiation and progression between different groups were analyzed using SPSS program. Analysis of variance test (ANOVA) and Tukey post hoc tests were used to compare the differences between the groups.

RESULTS

Table 2 summarizes the number of sound, decalcified, and carious teeth in all the groups. All the teeth in the positive control (Group 1) developed cavitations. All experimental groups were free from cavitations. For the decalcification, Group 3 (50% Fe) showed the least number of teeth (one tooth) with decalcification followed by Group 2 (100% Fe) which had 3 teeth with decalcification.

Table 2. Summary of total number (%) of teeth with sound, decalcified, and cavitated teeth (N=12/group)

Groups	Sound ^{1,2}	Decalcified ¹	Cavitated ²
Group 1 (with bacteria but no iron)	0 (0.0)	12 (100)*	12 (100)
Group 2 (with bacteria, 100% iron)	9 (75)	3 (25)	0 (0.0)
Group 3 (with bacteria, 50 % iron)	11 (91.7)	1 (8.3)	0 (0.0)
Group 4 (with bacteria, 25% iron)	5 (41.7)	7 (58.3)	0 (0.0)
Group 5 (with bacteria, 12.5 % iron)	3 (25)	9 (75)	0 (0.0)

- Group 1 vs Groups 2, 3, 4, 5
Group 2 vs Group 5
Group 3 vs Group 4
Group 3 vs Group 5

$P < 0.001$

$P = 0.014$

$P = 0.009$

$P = 0.001$

- All pairs were significant ($P < 0.001$)

* Total number is 12, 12 is placed here for comparison

Table 3. Analysis of variance for starting day of decalcification and starting day of cavitation

		Sum of Squares	df	Mean square	F	P-value
Starting day of decalcification	Between groups	5539.233	4	1384.808		
	Within groups	1709.500	55	31.082	44.554	<0.001*
	Total	7248.733	59			
Starting day of cavitation	Between groups	41.667	4	10.417		
	Within groups	26.917	55	0.489	21.285	<0.001*
	Total	68.583	59			

*Statistical significance for starting day of decalcification and cavitation

Group 1 vs Groups 2,3,4, and 5 ($P < 0.0001$)

Group 3 vs Group 5 ($P < 0.05$)

Table 3 compares the difference between different groups' mean of starting days of decalcification and cavitation. ANOVA showed that there were significant differences among the groups for starting days of decalcification and cavitation ($P < 0.001$). Post Hoc Tukey test revealed that Group 1 was significantly different from other groups ($P < 0.001$). Furthermore, Group 3 was significantly different from Group 5 ($P < 0.05$) (Table 3).

DISCUSSION

The objective of this *in vitro* study was to examine the effect of different concentrations of iron on the initiation of dental caries in human teeth. We used the *in vitro* caries model for the ease of control on the factors affecting dental caries. In addition, result from this *in vitro* study may help in the design of an *in vivo* future study.

Results from this laboratory study have demonstrated the cariostatic effect of iron (ferrous sulfate) at different concentrations 100%, 50%, 25% and 12.5%. The best effect of iron was with 50% concentration which showed 91.7% fewer lesions followed by 100% concentration which showed 75% fewer caries lesions. These percentages decreased to 41.7%

and 25% fewer carious lesions in groups 4 and 5, respectively.

Our results confirm the findings of previous studies that showed a cariostatic effect of ferrous sulfate.^{11,12} It was suggested by McClure¹⁵ that anion might influence the cariostatic effect of iron. In addition, it has been well documented that reactive oxygen species cause damage to various biomolecules. When the metal ion is bound to an enzyme, it is involved in the oxidation-reductions. The locally generated oxygen species may react at specific sites with the enzyme impairing its activity.¹⁶ Ferrous ions react with molecular oxygen by oxidation and yield superoxide radical ions. By dismutation reaction, the superoxide radical ions produce hydrogen peroxide. The ferrous ion reacts with hydrogen peroxide to produce hydroxyl radical ion via the Fenton reaction.

In this study, FRE-IN-SOL iron supplement, which contains ferrous sulfate, was used because it is the available iron supplement in King Abdul-Aziz University Hospital (KAUH) and King Khalid University Hospital (KKUH). We aimed to use the same iron supplements prescribed by pediatricians in KKUH and KAUH.

As this study was a pilot one, the period that the study was carried out was 29 days. This time allowed the caries initiation and some cavitations. Future studies may need to extend the time over 29 days. In addition, other iron supplement products could be used in future studies to assess the effect of each supplement.

In this pilot study, visual examination was used to diagnose decalcification and cavitation. Traditionally, dental caries can be diagnosed by visual examination, tactile examination (by explorer), radiographic examination and/or by a combination of the above methods.¹⁷ Previous studies have used electron

microscope to diagnose the caries in similar *in vitro* studies. Due to the number of the teeth and the objectives of the study, sectioning the teeth at different intervals was not possible. In a recent study, laser-based diagnostic system (DIAGNOdent) was compared to visual examination for the diagnosis of caries.¹⁸ The results showed that DIAGNOdent was helpful to diagnose actual cavitation, while visual examination was important to diagnose early caries signs (white spot lesion as decalcification). Although visual examination may be subjective, recent study showed the importance of using visual examination with other advanced techniques (DIAGNOdent) for the diagnosis of decalcification and dental caries.¹⁹ Visual examination was used in this study because decalcification is easy to diagnose after drying the tooth.

In the study, staining was observed in some teeth. A previous study had shown that daily inorganic iron, not organic, may cause tooth-staining.²⁰ In another study, staining was observed with both ferrous and ferric iron.⁸ The presence of extrinsic staining on the enamel surface of teeth receiving ferrous sulfate indicates the precipitation of inorganic iron.

CONCLUSION

The results of this *in vitro* study enable us to conclude that iron plays a cariostatic role in the development of dental caries in artificial dental caries experiments regardless of its concentration.

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