

Comparison of the Effect of Three Types of Iron Drops on Surface Roughness of Deciduous Teeth in a Simulated Cariogenic Environment

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Abstract

Objective: Iron deficiency anemia is among the most common types of childhood anemia. According to the World Health Organization, approximately 5 billion people were suffering from iron deficiency anemia worldwide in 2001. Aside from causing anemia, iron deficiency can negatively affect the physical and mental development of children and adolescents. Several studies have discussed the consequences of inadequate iron intake among which we may name changes in deciduous teeth. Considering the importance of iron supplementation, the present in-vitro study aimed at assessing the surface roughness of deciduous teeth following exposure to three different iron drops.

Methods: This in-vitro experimental study was conducted on 90 sound anterior deciduous teeth that were divided into 6 groups. After surface preparation, the teeth were placed in cariogenic environment. Different ferrous sulfate compounds were added to 4 media and the remaining two groups were considered as positive and negative controls. Fourteen days later, the specimens were removed from the media, sectioned labiolingually, polished and enamel and dentin microhardness were evaluated. The mean microhardness value for the 15 specimens in each group was recorded. ANOVA was applied for comparison of data and LSD test was used for multiple comparisons.

Results: No statistically significant differences were found in enamel microhardness of the 6 understudy groups. The mean microhardness of dentin was significantly different in the three understudy depths. Dentin microhardness immediately below the DEJ, at 250 Mm distance from the DEJ and at 450 Mm distance from the DEJ was (kgf/m^2) 68.72 (10.00), 67.75 (8.75) and 68.75 (11.86) in group 1, 69.22 (12.46), 73.06 (9.36) and 69.29 (8.01) in group 2, 68.533 (12.27), 64.63 (10.64) and 69.64 (10.15) in group 3, 83.033 (11.22), 71.68 (16.01) and 70.88 (17.60) in group 4, 60.080 (9.83), 63.52 (14.46) and 65.49 (11.20) in group 5 and 91.91 (43.76), 88.62 (20.47) and 85.04 (26.56) in group 6 ($p=0.001$ for all three), respectively. Pair-wise comparison of groups revealed that the mentioned difference is due to the statistically significant differences between group 6 and other groups and the remaining groups were not significantly different.

Conclusion: This study showed that iron supplementation had no effect on demineralization of tooth structure.

Key words: Cariogenic medium, Deciduous teeth, Iron deficiency, Iron drop, Anemia, Microhardness,

Please cite this article as follows:

Eskandarian T, Motamedifar M, Hekmatfar S, Tamaddon MA. Comparison of the Effect of Three Types of Iron Drops on Surface Roughness of Deciduous Teeth in a Simulated Cariogenic Environment. J Dent Sch 2013; 31(1):15-22.

Received: 26.08.2012

Final Revision: 17.02.2013

Accepted: 06.03.2013

Introduction:

Iron deficiency anemia is among the most common types of childhood anemia. The World Health Organization has reported the prevalence

of iron deficiency anemia in the world to be 30% (1). But according to local statistics, the prevalence of iron deficiency anemia in pregnant women, infants and children in developing countries is 50-60% (2). Iron deficiency has

adverse effects on physical and mental development of children and adolescents. Iron deficiency leads to behavioral and cognitive performance problems in children. Iron deficient children in their early years of life score low in different mental and motor performance tests(2, 3).

Various compositions of oral iron supplements are available in the market and prescribed for iron deficient patients. At present, in our country Iran, 15 drops of iron are administered orally in 6-24 month old babies as a national health policy to prevent iron deficiency anemia. Despite the importance and high costs of this act, inadequate or irregular intake of iron supplementation has been widely reported. Various reasons have been suggested for this issue among which, we may name the changes in deciduous teeth as the result of iron intake (4). Black discoloration (stains) of deciduous teeth due to the consumption of iron supplements is among the most important reasons for patient visits to a pediatric dentist.

Several human and animal studies have evaluated the potential effect of iron on development and progression of dental caries, enamel decalcification, concentration of saliva, stains, oral microbial flora and many other important factors reporting positive, negative and sometimes controversial results. Martinhon in 2006 evaluated the effect of ferrous sulfate on the reduction of demineralization of blocks of enamel and changing the ionic composition of the formed biofilm. He demonstrated that in a cariogenic environment, ferrous sulfate significantly decreased enamel demineralization and the percentage of surface microhardness change in enamel blocks (5).

Buzalaf *et al.* in 2006 evaluated the potential effect of increasing concentrations of iron on inhibition of demineralization of bovine enamel powder. They noticed a dose-dependent response in reducing the dissolution of mineral content of enamel powder and by increasing the concentration of iron ions the concentration of

phosphate ions in acetic acid decreased. The highest inhibitory effect (degree of protection) of iron was observed at 15 mmol/lit concentration. In higher concentrations, iron had no extra effect on inhibiting the dissolution of enamel powder (6).

Sales-Peres *et al.* in 2007 evaluated the effect of a mouth rinse containing iron on enamel and dentin erosion in an in-situ study. They used Coca Cola to cause erosion and found that rinsing 10 ml of a mouthwash containing 10 mmol/lit ferrous sulfate for one minute after an erosive attack significantly decreased the erosion, wear and changes in surface microhardness of enamel. They also noticed a significant reduction in dentin wear after the application of mouth rinse. However, no significant change was detected in surface microhardness of dentin (7).

Kato *et al.* in 2009 introduced iron varnish for the first time and evaluated its effect on bovine enamel erosion. Their obtained results revealed that the iron varnish could prevent the dissolution of enamel in presence of erosive beverages. Also, reduction in enamel wear was significantly different following the application of iron varnish versus fluoride varnish (8).

Thakib in 2003 assessed the cariostatic effect of four different iron supplements (Feromin, Fer in Sol, Ferose and Ferotonic at two concentrations of 50% and 100%). He used *S. mutans* culture medium to simulate carious lesions. The results showed that except for Ferose, the remaining three iron supplements did not have a cariostatic effect (9).

Bueno in 2010 compared the effect of iron gel with or without fluoride on bovine enamel erosion. The results demonstrated that iron gel with or without fluoride could prevent enamel dissolution by approximately 40% (10).

Considering the importance of iron supplementation with the use of iron drop and lack of studies regarding its effect on the structure of deciduous teeth, the present study

was designed aiming at comparing an Iranian iron drop with a foreign-made product and also an iron drop with Xylitol in terms of changing the microhardness of tooth surface.

Methods:

In this in-vitro experimental study, a total of 90 anterior deciduous teeth extracted because of space discrepancy, mobility or trauma were selected. All teeth were evaluated for presence of carious lesions, developmental anomalies, enamel cracks, discoloration and internal or external pathological changes in the pulp chamber. The teeth were excluded from the study if they met any of the mentioned exclusion criteria. After collection, all teeth were stored in distilled water at room temperature until the conduction of experiment. For preparation of specimens, the teeth were dissected at the CEJ and the pulpal residues in the pulp chamber were completely removed. Pulp chamber was then filled with composite resin. A label was placed on the buccal surface of prepared teeth measuring 0.4x0.4 cm. The entire area surrounding the label was coated with nail polish. The label was then removed and the glue residues were washed off with a gauze and water (Figure 1).



Figure 1- Preparation of a rectangular area (window) at the buccal surface of teeth measuring 0.4x0.4 cm.

The teeth were then immersed in 10% formaldehyde with a neutral pH for 30 days to become sterilized.

The prepared cariogenic environment included 3.7 g Brain Heart Infusion (BHI), 0.5 g yeast extract, 2 g sucrose and 1 g glucose dissolved in 100 ml of distilled water; 100 microliter of fresh culture (18-24 h) of standard *S. mutans* (ATCC 25175) was added to this medium. PH of the medium was adjusted to 4. A test tube was allocated to each specimen and the respective products along with the culture medium were transferred to the test tube (11).

The understudy products were ferrous sulfate iron drop (Kharazmi Co., Iran), Irovit iron drop (Vitane Pharmaceuticals, Inc. Germany) and an iron drop manufactured in Shiraz School of Pharmacy containing Xylitol (Table 1).

Table 1- Composition of the understudy products

Product name	Composition	Manufacturing company and country
Kharazmi iron drop	125 mg ferrous sulfate+ sodium saccharin as sweetener (each ml contains 25 mg iron ion)	Kharazmi Co., Iran
Irovit iron drop	75 mg ferrous sulfate + strawberry flavor (each ml contains 15 mg iron ion)	Vitane Pharmaceuticals Inc., Germany
Iron drop containing Xylitol	125 mg ferrous sulfate+ Xylitol as the sweetener (each ml contains 25 mg iron ion and 1 g Xylitol)	Shiraz School of Pharmacy, Iran
Ferrous sulfate	FeSo ₄ *7H ₂ O M=278.02 g/m	Alfa Aesar Co., Germany

Six groups of 15 were prepared as follows:

Group 1: 25 ml of cariogenic medium+ 1 ml of iron drop (Kharazmi Co.)

Group 2: 25 ml of cariogenic medium + 1 ml of Irovit iron drop (Vitane Pharmaceuticals Inc.)

Group 3: 25 ml of cariogenic medium + 1ml of iron drop containing Xylitol (Shiraz School of Pharmacy)

Group 4: 25 ml of cariogenic medium + 1ml of ferrous sulfate (Merck, Germany)

Group 5: 25 ml of cariogenic medium

Group 6: 25 ml of culture medium without *S. mutans*

All groups were incubated in microaerophilic conditions at 37°C for 14 days. The media were refreshed every 48 h and specimens were transferred to a new medium. After completion of this time period, the teeth were removed from the media and prepared for microhardness evaluation (12). Microhardness indirectly shows the mineral content of enamel and dentin; thus, this test was used for assessment of changes in tooth surface. To determine microhardness, tooth surface at the window area was vertically divided into two segments. The obtained piece was embedded in acrylic resin in a way that the entire tooth surface except for the sectioned area was covered. Prepared specimens were polished with 600, 800 and 1200 grit abrasive paper discs. Final polishing and finishing was done using a solution containing 0.5 and 0.03 mM aluminum oxide particles and a special cloth (Figure 2).



Figure 2- Sectioned and polished tooth embedded in acrylic resin

Considering the structure of deciduous teeth, 25 g load was applied to tooth surface during 30 s (12,13). To determine microhardness, indentations were made at 200 Mm distance from the tooth surface in enamel, below the DEJ and at two points with 200 Mm distance in dentin using Vickers microhardness testing machine. Obtained data for microhardness of each specimen were recorded at 5 different depths. The mean of the obtained microhardness values for 15 specimens in each group was calculated. The mean values were analyzed in the 6 understudy groups using ANOVA. Multiple comparisons were made using LSD test.

Results:

Evaluation of microhardness of specimens yielded the following results:

No significant difference was found when the 6 groups were compared in terms of enamel microhardness at 200 Mm depth from the tooth surface ($p=0.211$). Although the microhardness value obtained in group 6 (culture medium without the *S. mutans*) was higher than in the remaining 5 groups, enamel microhardness at 400 Mm depth was not significantly different among the 6 understudy groups ($p=0.074$). At 400 Mm depth, the highest microhardness value was recorded in group 6. The microhardness of dentin at all measured points was significantly less than that of enamel (Table 2).

Table 2- The mean enamel and dentin microhardness (Kgf/m²) of deciduous teeth after immersion in medium

Groups	Mean±SD				
	200 Mm depth	400 Mm depth	Below the DEJ	At 250 Mm distance from the DEJ	At 450 Mm distance from the DEJ
Group 1	392.57 (185.61)	273.29 (175.62)	68.72 (10.00)	67.75 (8.75)	68.75 (11.86)
Group 2	317.60 (72.01)	201.90 (57.62)	69.22 (12.46)	73.0 (9.36)	69.29 (8.01)
Group 3	350 (107.03)	265.71 (93.17)	68.533 (12.27)	64.63 (10.64)	69.64 (10.15)
Group 4	351.93 (164.42)	201.27 (82.07)	83.033 (11.22)	71.68 (16.01)	70.88 (17.60)
Group 5	337.80 (86.70)	237.93 (62.75)	60.08 (9.83)	63.52 (14.46)	65.49 (11.20)
Group 6	432.53 (147.71)	285.20 (78.42)	91.91 (43.76)	88.62 (20.47)	85.04 (26.56)

In all three tested points in dentin, the highest and lowest microhardness values belonged to

group 6 and group 5, respectively. A statistically significant difference was noted in dentin

microhardness below the DEJ and at 250 Mm and 450 Mm depth from the DEJ ($p=0.001$ for all three). Pair-wise comparison of groups revealed that this difference was due to group 6 which had statistically significant differences with other groups. The remaining groups had no statistically significant difference with one another (Diagram 1).

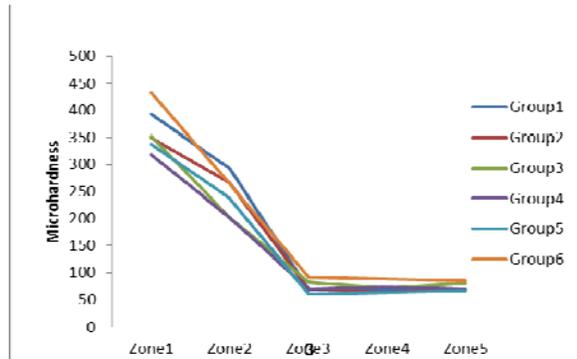


Diagram 1. Microhardness of deciduous teeth in the 6 understudy groups at 5 different depths

Discussion:

Iron deficiency and subsequent anemia are among the common public health problems worldwide especially in developing countries. High prevalence of iron deficiency anemia in children and its correlation with physical and mental disorders observed in different studies highlight the importance of preventing this condition (2).

For treatment of iron deficiency anemia several methods of iron supplementation are used; among which, oral administration of iron drops and syrups is the most common approach for prevention of iron deficiency in our country, Iran. Consumption of iron-containing compounds causes black discoloration of deciduous teeth. This issue has been confirmed in studies by Mehran (2009), Makarem (2006) and Shojaipour (2010) on discoloration of deciduous teeth exposed to iron drops (14-16).

This in-vitro study aimed to evaluate the effect

of three different types of iron drop on surface changes of deciduous teeth. At present, different types of iron drops with different concentrations and sometimes in combination with minerals and/or vitamins are available in the market. In this study, Kharazmi and Irovit iron drops and a new product made in Shiraz School of Pharmacy were used. Kharazmi and Irovit iron drops are available in the market and accessible by the public. They contain ferrous sulfate without other supplements such as zinc or vitamins. In Kharazmi iron drop, saccharin is added as a sweetener; which is a non-cariogenic compound. The iron drop manufactured in Shiraz School of Pharmacy was used to evaluate the effect of Xylitol as sweetener in combination with ferrous sulfate.

No statistically significant difference was detected between the 6 groups in enamel microhardness at two different depths after immersion in different culture media. Also, our study results showed that changes in microhardness were significantly different between cariogenic and non-cariogenic media but presence of different iron compounds in the media and positive control group did not cause a significant change in degree of demineralization. Kato *et al.* in 2009 (8) and Bueno *et al.* in 2010 (10) evaluated the effect of iron varnish and iron gel on enamel dissolution, respectively and demonstrated that ferrous sulfate interferes with enamel demineralization and prevents enamel dissolution. Difference in results may be due to the fact that the used medium in the mentioned studies was an acidic environment causing demineralization in a fashion similar to erosion. Furthermore, the concentration of ferrous sulfate used in both studies was 10 mmol/lit.

Our obtained results were in contrast to those of Martinhol *et al.* in 2006 (5) and Sales-Peres *et al.* in 2007 (7). Martinhol *et al.* in 2006 performed an insitu study and used 15 mmol/lit concentration of ferrous sulfate. They stated that reduced demineralization of enamel blocks in

presence of ferrous sulfate was due to the formation of a ferric phosphate layer on enamel that is formed by the reaction of iron ion and phosphate ion on the enamel surface. Sales-Peres *et al.* in 2007 used 10 mmol/lit concentration of ferrous sulfate and showed that application of ferrous sulfate mouthrinse after the development of erosion can reduce the dissolution of enamel and dentin structure (7).

The main difference between our study and the previous ones conducted on the effects of ferrous sulfate is the type, composition and concentration of iron used; which seems to be the major factor causing a difference in the obtained results. The aim of the present study was to determine the role of iron supplementation in changing the structure of deciduous teeth. Supplements were used in prepared therapeutic dosages. Kharazmi iron drop in each ml contained 25 mg iron ion. Irovit in each ml contained 15 mg iron ion. The iron drop manufactured in Shiraz School of Pharmacy contained Xylitol and pure ferrous sulfate added to groups 3 and 4 in each ml contained 25 mg iron ion. Buzalaf *et al.* in 2006 evaluated the potential effect of increasing concentrations of iron on inhibition of demineralization of bovine enamel powder and observed minimum reduction (10%) at 0.625 mmol/lit concentration and maximum reduction in enamel dissolution at 15 mmol/lit concentration in an acidic environment (6).

Only two similar studies have evaluated the effect of iron supplements on initiation of caries. Al Shalan *et al.* in 2006 stated that Fer in Sol iron supplement was effective for initiation of dental caries (17). Also, Thakib *et al.* in 2003 evaluated 4 iron supplements with 50 and 100% concentrations and showed that all the under study supplements had anti-caries properties except for one type that contained maltose (9). Similarly, Thakib in 2003 used a culture medium containing *S. mutans* for evaluation of the effect of iron supplements.

However, caries assessment in his study was done with visual observation and use of an explorer that has higher error probability than microhardness. Difference between our study results and those of Thakib (2003) is explained by the method of assessment of specimens, type of used supplements and their concentration. Supplements used in this study had no cariogenic sugar compound for elimination of confounding factors.

Xylitol is an alcohol sugar and its effect on increasing salivary flow and enhancing tooth remineralization has been documented in several studies. Furthermore, Xylitol is not fermentable in the oral environment and reduces the number of *S. mutans* colonies in dental plaque (18-20). In 2009, Milgrom conducted a study on 118 9-15 month babies that took Xylitol syrup. The obtained results showed that consumption of 8 g Xylitol daily in two or three meals could reduce childhood caries by 70%. In 2008, Milgrom in an in-vitro study evaluated the effect of Xylitol on dental biofilm. He created dental biofilm containing 6 bacterial strains and exposed it to 1% and 3% concentrations of Xylitol. The results revealed that Xylitol clearly inhibited biofilm formation in vitro and it can be successfully used for prevention of oral disease due to biofilm formation (21). The results of the mentioned study are in agreement with our findings. The iron drop manufactured in Shiraz School of Pharmacy contained 1 g/ml Xylitol. However, presence of Xylitol did not cause a statistically significant difference between groups. Furthermore, Badet in 2008 (22), Oscarson in 2006 (23) and Honkala in 2006 (24) emphasized the importance of concentration of Xylitol for its effectiveness against *S. mutans* and concluded that 1 g concentration of Xylitol was not effective for caries prevention.

No similar study was found in the literature review to compare their results with ours. It seems that iron supplements can be prescribed with no concern of the demineralization of tooth

structure. Considering the fact that the present study was conducted under in-vitro conditions, further studies are required on human and animal models to determine the exact mechanism of the effect of iron compounds on tooth structure.

Conclusion:

The obtained results demonstrated that iron supplements had no effect on decreasing or increasing the demineralization of tooth

structure in a cariogenic environment.

Acknowledgement

This study was part of the doctoral thesis of Dr. Somayeh Hekmatfar (#90-5774). The authors would like to express their gratitude to the Research Deputy of Shiraz University of Medical Sciences for financially supporting this study.

Conflict of Interest: “None Declared”

References:

1. Silva DG, Priore SE, Franceschini Sdo C. Risk factors for anemia in infants assisted by public health services: the importance of feeding practices and iron supplementation. *J Pediatr (Rio J)* 2007; 83: 149-156.
2. World Health Organization. Iron deficiency anemia. Assessment prevention and control. A guide for program manager. Geneva, Switzerland 2001.
3. Novkes C, Van den Bosch C, Bundy DAP. The effects of iron deficiency and anemia on mental and motor performance educational achievement and behavior in children. An annotated bibliography. International nutritional anemia consultative group. U.S. 1998.
4. Mozaffari-Khosravi H, Hosseinzadeh M, Mozaffari-Khosravi V. The Study of Iron-Drop Supplementation Status on 6-24 Month Infants in Yazd Health Centers. *Scientific Journal of Yazd Medical University of Medical sciences* 2010; 29(2,3): 56-68.[Persain]
5. Martinhon CC, Italiani M, Padilha P, Bijella B, Delbem B, Buzalaf R. Effect of iron on bovine enamel and on the composition of the dental biofilm formed “in situ”. *Oral Biology* 2006; 51: 471-475.
6. Buzalaf MA, de Moraes Italiani F, Kato MT, Martinhon CC, Magalhães AC. Effect of iron on inhibition of acid demineralization of bovine dental enamel in vitro. *Arch Oral Biol* 2006; 51: 844-848.
7. Sales-Peres SH, Pessan JP, Buzalaf MA. Effect of an iron mouthrinse on enamel and dentine erosion subjected or not to abrasion: an in situ/ex vivo study. *Arch Oral Biol* 2007; 52: 128-132.
8. Kato MT, de Moraes Italiani F, de Araújo JJ, Garcia MD, de Carvalho Sales-Peres SH, Buzalaf MA. Preventive effect of an iron varnish on bovine enamel in vitro. *J Dent* 2009; 37: 233-236.
9. Thakib A. AL- Shalan. In vitro cariostatic effect of various iron supplements on the initiation of dental caries. *Saudi Dent J* 2003; 21: 117-122.
10. Bueno MG, Marsicano JA, Sales-Peres SH. Preventive effect of iron gel with or without fluoride on bovine enamel erosion in vitro. *Aust Dent J* 2010; 55: 177-180.
11. Hara AT, Queiroz CS, Giannini M, Cury JA, Serra MC. Influence of the mineral content and morphological pattern of artificial root caries lesion on composite resin bond strength. *Eur J Oral Sci* 2004; 112: 67-72.
12. Marquezan M, Corrêa FN, Sanabe ME, Rodrigues Filho LE, Hebling J, Guedes-Pinto AC, *et al.*

- Artificial methods of dentine caries induction: A hardness and morphological comparative study. *Arch Oral Biol* 2009; 54: 1111-1117.
13. Hosoya Y, Marshall SJ, Watanabe LG, Marshall GW. Microhardness of carious deciduous dentin. *Oper Dent* 2000; 25: 81-89.
 14. Mehran M, Mohammadi Basir M, Jafari S. Effect of two kinds of iron drops on the discoloration, atomic absorption and structural changes of primary teeth enamel. *J Dent Med* 2009; 21: 290-299. [Persain]
 15. Makarem A, Orafai H, Shabzندهdar M, Khashayarmanesh Z, Ebrahimzadeh S. Comparison of primary enamel discoloration caused by the use of three different iron drops. *Journal of Dentistry. Mashhad University of Medical Sciences* 2006; 30: 247-254. [Persain]
 16. Shojaipour R, Khazaeli Pharm D, Mahmodi T. Adsorption rate of Iron on to primary incisor teeth following the application of three iron drops. *J Kerman Univ Med Sci* 2010; 17: 42-48. [Persain]
 17. Al-Shalan, Takib A, Al-Askar, Amal. In vitro effect of different concentrations of iron on the initiation of dental caries: pilot study. *Saudi Dent J* 2006; 18: 86-90.
 18. Söderling E, Trahan L, Tammiala-Salonen T, Häkkinen L. Effects of xylitol, xylitol-sorbitol, and placebo chewing gums on the plaque of habitual xylitol consumers. *Eur J Oral Sci* 1997; 105: 170-177.
 19. Maguire A, Rugg-Gunn AJ. Xylitol and caries prevention: is it a magic bullet? *Br Dent J* 2003; 194: 429-436.
 20. Assev S, Stig S, Scheie AA. Cariogenic traits in xylitol-resistant and xylitol-sensitive mutans streptococci. *Oral Microbiol Immunol* 2002; 17: 95-99.
 21. Milgrom K, Ly KA, Rothen M. Xylitol and its vehicles for public health needs. *Adv Dent Res* 2009; 21: 44-47.
 22. Badet C, Furiga A, Thébaud N. Effect of xylitol on in vitro model of oral biofilm. *Oral Health Prev Dent* 2008; 6:3 37-341.
 23. Oscarson P, Lif Holgerson P, Sjöström I, Twetman S, Stecksén-Blicks C. Influence of a low xylitol-dose on mutans streptococci colonization and caries development in preschool children. *Eur Arch Paediatr Dent* 2006; 7: 142-147.
 24. Honkala E, Honkala S, Shyama M, Al-Mutawa SA. Field trial on caries prevention with xylitol candies among disabled school students. *Caries Res* 2006; 40: 508-513.