A Spectroscopic and Surface Microhardness Study on Enamel Exposed to Beverages Supplemented with Lower Iron Concentrations

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Objectives: *This study aimed to compare the in vitro mineral loss and surface microhardness (SMH) changes in human enamel specimens following supplementation of acidic carbonated beverages with low iron concentrations than when treated without.* ***Study Design:*** *180 enamel blocks each from primary and permanent teeth were prepared and equally subdivided (n=10) for their respective treatments in Group 1 (Coca Cola and Sprite without iron supplementation) and Group 2 (beverages supplemented with 2/5mmol/L* $FeSO_4.7H_2O$*). Following initial SMH estimation, the blocks were subjected to 3 treatment cycles of 5/20 minute incubation periods, equally interspaced by a 5-min treatment in artificial saliva. The calcium and phosphate released after each cycle were analyzed spectrophotometrically and the final SMH was recorded. The results were tested using student's T test, One-way ANOVA and Kruskal Walli's test (p<0.05).* ***Results:*** *Two and five mmol/L* $FeSO_4.7H_2O$ *supplementation produced a highly significant SMH change and calcium and phosphate reduction than when treated without (p<.0005). Both the enamel specimens showed similar patterns of mineral loss and SMH reduction, with pronounced effects in the twenty minute incubation cycles.* ***Conclusion:*** *Our results suggest that 2mmol/L* $FeSO_4.7H_2O$ *supplementation to acidic beverages is beneficial in reducing mineral loss and preserving surface microhardness of human enamel.*

Key words*: Dental enamel. Aerated beverages. Tooth erosion. Surface microhardness. Iron.*

INTRODUCTION

Dental hard tissue loss is caused by a number of factors, including dental caries, trauma, and, increasingly tooth wear - which can occur by abrasion, attrition, and erosion[1]. Carbonated drinks have an inherent acidity due to acids added to elicit better taste and counteract sweetness; these acids include phosphoric acid and citric acid[2]. The consumption of soft drinks such as acidic fruit juices, artificially sweetened fruit drinks, and carbonated beverages are the risk factors most significantly related to this dental hard tissue defect[2]. Recent updates have shown that the prevalence of dental erosion in children and adolescents due to the increased consumption of carbonated drinks scores up to 65% and 62% for the primary and permanent dentitions, respectively[3,4].

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Supplementation of soft drinks with ions, such as calcium, phosphate, fluoride and iron in order to make these beverages more saturated in respect to the dental minerals, thus reducing their erosive potential has been studied[5,6]. The addition of calcium and phosphate has shown favorable results[5], but the addition of fluoride has a good effect only when toxic concentrations are used[6]. Ionic iron is known to have cariostatic properties[7] and inhibits enamel dissolution in an erosive/abrasive environment[8]. Some preliminary studies have speculated that the supplementation of foods and beverages with iron could work as an alternative strategy to inhibit the dissolution of bovine enamel powder under acidic conditions reducing not only dental caries, but also dental erosion[9-12]. Many hypothetical mechanisms have been proposed to explain this protective effect, but the precise mechanism still remains a challenge.

A study by Kato MT *et al*[13] (2007) has proven that a beverage containing 10mmol/L $FeSO_4.7H_2O$ can reduce the dissolution of bovine dental enamel in-vitro. However, there has been a need for a study based on the effect of lower iron concentrations on enamel following acid challenges, specifically focusing on human enamel specimens of both primary and permanent teeth. Thus, this in vitro study evaluated the mineral and surface microhardness loss of human permanent and primary enamel exposed to beverages supplemented with low iron concentrations compared to the original beverages.

MATERIALS AND METHOD

100 recently extracted human deciduous incisors and young permanent premolars in children requiring extraction for orthodontic treatment were collected and stored as per the Occupational Safety and Health Act regulations[14]. Teeth having no signs of attrition,

abrasion, erosion, fluorosis or demineralization on its enamel surfaces were included. The Iron solutions used included 2 and 5mmol/L of Ferrous sulphate[15]. Two acidic carbonated beverages (Hindustan Coca Cola beverages Pvt. Ltd, Bangalore, India) namely, Coca cola® and Sprite® were chosen for the experimentation. The pH of Coca cola® and Sprite® were 2.58 and 2.98 respectively, measured using a pH meter (Orion 900A, Boston, MA, USA). Mineral water (Kinley, Coca Cola beverages Pvt. Ltd, Bangalore) was used as the control. Artificial saliva was prepared similarly to that described by Zero *et al*[16].

Estimation of Iron in the beverages

The quantity of iron already present in the carbonated beverages was estimated using the Iron Chromazurol method[17]. A very minimal concentration of 0.1059mg/L was noticed in Coca Cola®, while not in Sprite® and mineral water.

180 Enamel blocks each from primary and young permanent teeth were prepared of dimensions 4mm x 3mm x 2mm[18,19] using double sided diamond coated discs (Galaxy™ 19 mm diamond coated discs). The enamel surface of the specimens was ground flat with water-cooled carborundum discs (600 and 1200 grades of Al2O3 papers; Buehler) and polished with wet felt paper, resulting in the removal of about 100µm depth of enamel, controlled with a micrometer[20]. The specimens were then weighed and the quantity of carbonated beverage to be exposed was based on the criteria followed by Kato MT *et al*, where 10mmolL^{-1} of the beverage was exposed to 1mg enamel [15]. This achieved the standardization of beverage treatment in the blocks.

Ethical approval and protocol authorization for the study was provided by the Institutional committee for ethics and research, affiliated to the Rajiv Gandhi University of Health Sciences, Bangalore. An initial surface microhardness measurement for each enamel block was recorded using a Knoop hardness tester[21,22](Clemex, Model MMT-X7, Matsuzawa Co. Ltd, Japan), 10sec, 50gf load (five indentations, equally spaced, over the outer enamel surface); performed at the National Institute of Technology, Mangalore, Karnataka, India. The mean of the obtained values was considered as the existing surface microhardness (KHN).

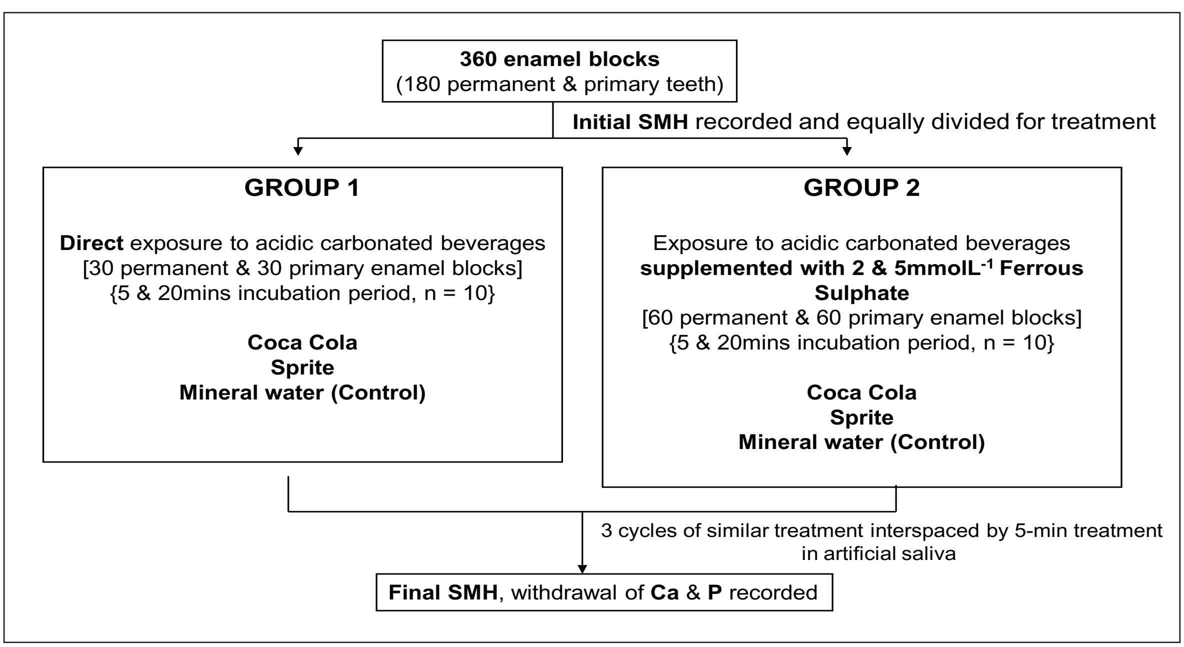
The primary and permanent enamel blocks were randomly divided into 60 and 120 for their respective treatments in *Group 1* (enamel blocks + acidic carbonated beverages without iron supplementation) and *Group 2* (enamel blocks + acidic carbonated beverages supplemented with 2/5 mmol/L Ferrous Sulphate). Ten enamel blocks each (n=10) were available for the treatment in every sub-category within the groups (Figure1).

One complete cycle of treatment consisted of a demineralization phase in 10mmolL^{-1} of the beverage per milligram of enamel for 5/20 minute incubation cycles under gentle agitation, followed by a remineralization phase in 10mmolL^{-1} of artificial saliva at 37°C for 5 minutes. The calcium and phosphate released from the enamel blocks after each of the 3 repetitive cycles of similar treatment[13] were analyzed spectrophotometrically (Spectrophotometer 106, Systronics, Serial no. 5493, Ahmedabad) by O-Cresolphthalein Complexone method[23] and Ammonium Molybdate method[24] respectively. Following 3 repetitive cycles of similar treatment, the enamel blocks were rinsed under de-ionized water and were assessed for their final surface microhardness. The results were finally subjected to statistical analysis using student's T test, One-way Anova and Kruskal Walli's test using the Statistical Package for Social Sciences (SPSS) version 15.00 and MS-Excel. $p<0.05$ was considered significant and confidence interval at 95%.

RESULTS

The etching effects of acidic carbonated beverages on enamel surface were observed within 5 minutes of contact, and were most pronounced after 20 minutes. This is evident in the present study from the withdrawal of calcium and phosphate witnessed in the repetitive cycles of exposure. The Kruskal Walli's test compared the average total reduction of calcium and phosphate in both the

Figure 1: **Study design**



groups, showing a greater loss with Group 1 than Group 2 (Tables 1 & 2). Also, a much pronounced reduction was observed in the experimental groups at the 20-minute incubation than the 5-minute incubation cycles ($p<.05$).

On treatment of the enamel blocks with the acidic carbonated beverages in Group 1, the results using the Paired T-test analysis showed an average reduction of surface microhardness by 55% and 56% in permanent teeth and that of 61% and 58% in the primary teeth was observed respectively with the treatment of beverages for a 5 min incubation after 3 cycles of treatment ($p <.0005$) (Table 3). An increased loss in SMH percent was observed with the 20 minute incubation cycle was evident by a reduction of 70.8% and 72.8% in the permanent teeth and that of 61.8% and 61.44% in the primary teeth respectively ($p<.0005$) (Table 4).

Table 1: **The average total withdrawal of calcium and phosphate in the 5-minute incubation category**

		Group 1	Group 2 (2mmolL^{-1} $FeSO_4$)	Group 2 (5mmolL^{-1} $FeSO_4$)
Permanent Enamel				
Cola beverage	**Ca**	38.3(±12.7)	21.7(±13.4)	24.4(±11.2)
	P	35.2(±9.6)	19.8(±8.2)	17.1(±9.4)
Acid beverage	**Ca**	29.1(±14.3)	14.5(±8.5)	12.4(±4.2)
	P	27.5(±11.7)	20.7(±11.4)	10.2(±4.4)
Mineral water	**Ca**	0	0.6(±0.35)	0.21(±0.07)
	P	0.3(±0.1)	1.2(±0.61)	0.3(±0.12)
Primary Enamel				
Cola beverage	**Ca**	16.6(±5.8)	10.8(±6.4)	8.4(±5.1)
	P	15.1(±6.4)	11.3(±4.9)	9.2(±3.8)
Acid beverage	**Ca**	9.1(±4.3)	4.2(±8.5)	3.7(±1.02)
	P	13.4(±5.8)	6.2(±3.4)	4.8(±2.3)
Mineral water	**Ca**	0	0.41(±0.23)	0.31(±0.22)
	P	0.12(±0.01)	0.33(±0.16)	0.81(±0.46)

[Mean ± SD; n = 10];

Ca = calcium, P = phosphate

The average total withdrawal of calcium (Ca) and phosphate (P) from permanent and primary enamel following 3 cycles of 5 minute incubation periods of group-wise treatment with the cola beverage, acid beverage (sprite) and mineral water is shown. ($p<.05$)

Table 2: The average total withdrawal of calcium and phosphate in the 20-minute incubation category

		Group 1	Group 2 (2mmolL^{-1} $FeSO_4$)	Group 2 (5mmolL^{-1} $FeSO_4$)
Permanent Enamel				
Cola beverage	**Ca**	43(±12.6)	29.1(±11.3)	31.7(±16.3)
	P	52.3(±13.7)	28.6(±12.7)	26.4(±14.3)
Acid beverage	**Ca**	38.5(±15.3)	26.7(±10.4)	18.7(±5.6)
	P	39.4(±18.2)	21.4(±12.8)	16.3(±9.5)
Mineral water	**Ca**	0.1(±0.02)	0.02(±0.01)	0.71(±0.43)
	P	0.2(±0.01)	0.72(±0.26)	1.01(±0.65)
Primary Enamel				
Cola beverage	**Ca**	31.3(±9.4)	21.3(±9.2)	12.7(±6.3)
	P	29.3(±12.4)	23.8(±11.6)	11(±5.92)
Acid beverage	**Ca**	24.1(±9.8)	11.1(±7.2)	3.8(±6.1)
	P	20.2(±8.2)	19.7(±7.3)	8.2(±5.6)
Mineral water	**Ca**	0	0.81(±0.56)	0.77(±0.37)
	P	0.21(±0.05)	0.63(±0.38)	0.47(±0.25)

[Mean ± SD; n = 10];

Ca = calcium, P = phosphate

The average total withdrawal of calcium (Ca) and phosphate (P) from permanent and primary enamel following 3 cycles of 20 minute incubation periods of group-wise treatment with the cola beverage, acid beverage (sprite) and mineral water is shown. ($p<.05$)

The surface microhardness variations of enamel treated with mineral water (control) for the 5 and 20 minute incubation treatments showed no statistical difference (p>.05) (Tables 3, 4). Negligible loss of calcium and phosphate from the enamel blocks was also noticed with the control in both Groups 1 and 2 (Tables 1, 2), statistically assessed using the Kruskal Walli's test (p>.05).

Two and five mmol/L $FeSO_4.7H_2O$ when added to the acidic carbonated beverages in the presence of the enamel block (Group 2), a definite calcium and phosphate release was observed, but the values were less significant than Group 1 (p<.05) (Tables 1, 2). Reduction in surface microhardness with the same treatment in both the incubation periods with both primary and permanent teeth was seen, but generally showed a very high statistical significance when compared to Group 1 (p<.0005). This implies that the addition of both these concentrations of iron to beverages wouldn't completely halt the release of mineral ions from the tooth, but, would definitely reduce its erosive potential.

When the 2 and 5 mmol/L concentration of $FeSO_4.7H_2O$ were compared for the mean SMH changes on permanent enamel specimens using the T-test for independent samples in Group 2, no statistical significance was observed, as seen with Coca Cola (t=1.1, p=.313) and Sprite (t= -.545, p=.606) following the 5-minute incubation cycles; similarly, Coca cola (t= -1.861, p =.112) and sprite (t= -4.583, p=.065) following the 20-minute incubation cycles (Tables 3, 4). With the primary enamel specimens, the readings showed Coca cola (t=1.570, p=.168) and Sprite (t= -1.573, p=.167) following the 5-minute incubation cycles; similarly, Coca cola (t= -.532, p =.761) and sprite (t= -1.315, p=.237) following the 20-minute incubation cycles.

DISCUSSION

Several researches were carried out to supplement soft drinks with mineral ions, thus reducing their erosive potential[5,6]. Recent research on the supplementation of various concentrations of iron has proven it to be a plausible alternative[9-12]. Buzalaf *et al*[10] proved that the presence of Fe^{2+} reduced significantly the phosphate loss from enamel by 45%. A study by Kato *et al*[12] showed that the best effect regarding the prevention of enamel dissolution is reached when the coke is carbonated and iron is directly added to the beverage. Kato *et al* [13] also reported that iron at 10mmol/L can reduce the dissolution of dental enamel *in vitro* in the presence of Coke. The cariostatic effect of iron on enamel demineralization have been suggested to its participation in the remineralization of human enamel and in the nucleation of apatite, substitution of

Table 3: **Surface microhardness changes of enamel with beverages in the 5-minute incubation category**

5 min incubation	Initial SMH	Final SMH	SMH Loss (%)
Group 1 analysis			
Cola + Permanent enamel	410.2(±27.7)	175.38(±22.5)	55.26
Cola + Primary enamel	379.7(±13.8)	148.8(±13.1)	61.93
Sprite + Permanent enamel	402.3(±16.56)	169.6(±25.9)	56.44
Sprite + Primary enamel	413.3(±21.5)	163.17(±21.1)	58.86
M. water + Permanent enamel	415.7(±15.2)	414.5(±6.56)	0.18
M. water + Primary enamel	389.7(±23.1)	388.3(±7.4)	0.43
Group 2 analysis			
Permanent enamel			
Cola + 2 mmol/L F.sulphate	406.5(±24.8)	261.8(±21.2)	34.86
Cola + 5 mmol/L F.sulphate	403.8(±12.6)	280.75(±23)	31.53
Sprite + 2 mmol/L F.sulphate	410.3(±29.8)	257(±28.3)	36.84
Sprite + 5 mmol/L F.sulphate	399.3(±29.8)	240(±28.3)	38.92
M. water + 2 mmol/L F.sulphate	417(±12.6)	411.6(±8.2)	1.24
M. water + 5 mmol/L F.sulphate	398(16.8)	396.4(±10.1)	0.33
Primary enamel			
Cola + 2 mmol/L F.sulphate	412(±29.8)	232(±14.3)	43.25
Cola + 5 mmol/L F.sulphate	379(±13.3)	203.6(±30)	45.43
Sprite + 2 mmol/L F.sulphate	426(±29.8)	240.2(±28.3)	42.86
Sprite + 5 mmol/L F.sulphate	345.1(±14.3)	188(±17.8)	45.5
M. water + 2 mmol/L F.sulphate	413.4(±21.4)	411.4(±9.8)	0.53
M. water + 5 mmol/L F.sulphate	386.7(±16.6)	382.8(±7.6)	1.06

[Mean ± SD; n = 10];

SMH = Surface microhardness in Knoop hardness number

The surface microhardness (SMH) changes and the mean SMH loss percent from permanent and primary enamel following 3 cycles of 5 minute incubation of group-wise treatment with the acidic carbonated beverages and mineral water is shown. (p<.05) calcium in apatite, inhibition of demineralization and formation of a protective film on enamel surface[22]. Thus, the current study aimed at comparing the mineral loss and surface microhardness changes when cola beverages were supplemented with a further lower iron concentration than when treated without.

Both the experimental groups were subjected to 3 repetitive cycles of 5 and 20 minute incubation periods, equally interspaced by a 5-minute treatment in artificial saliva. Considering the remineralization potential of saliva on acid attacked enamel surfaces[18] and its increased secretion upon predominantly activated autonomic nerves due to taste or chewing stimuli[25], we subjected the enamel blocks to artificial saliva following the end of a cycle. The method of assessing the mineral loss could be criticized, as the contact time between the enamel and beverage is 15 minutes for the 5-minute incubation cycles and 1 hour for the 20 minute incubation cycles. However, a child does not sip the entire beverage in a single swallow and would rather take several sips of the drink, the inter-duration, which may be shorter or longer. Sometimes, children consume more than one can of beverage a day. Hence, contact times of an hour, or even more, may be more realistic than shorter duration[26]. Thus, the subjection of the beverages to enamel to the 5 and 20 minute inter duration incubation cycles in our study simulated an in-vivo condition of intermittent drinking pattern in children.

As dissolution of enamel occurs at a critical pH of around 5.5, the present study shows that commercially available beverages have the potential to erode enamel due to their comparatively low pH's. This is evident in the present study from the withdrawal of calcium and phosphate observed following the repetitive cycles of exposure in Group 1 analysis (Tables 1, 2). But, the reduced loss of minerals noticed with the primary enamel than permanent can be related to its reduced inorganic content[27].

As attention is directed nowadays towards safe drinking water due to the increased incidence of water-borne diseases in Southern India, the general population at large consumes commercially available mineral water, most commonly *Kinley*, the control product used in this study. The same is a product of the Coca Cola Company, similar to the other experimental beverages used in this study. The product information mentioned only the presence of sodium as 0.3mg/100ml and magnesium as 0.1mg/100ml, while, the carbohydrate, sugar, protein and fat as zero grams. Thus, the selection of mineral water as the control achieved standardization, by being a product from the Coca Cola Company as the other tested products and was also found not to interfere with the calcium and phosphate levels during the estimation process.

The mineral loss of this *in vitro* study must be interpreted with a certain degree of caution, as they will tend to over estimate the

Table 4: **Surface microhardness changes of enamel with beverages in the 20-minute incubation category**

20 min incubation	Initial SMH	Final SMH	SMH Loss (%)
Group 1 analysis			
Cola + Permanent enamel	411(±27.7)	116.5(±26.2)	70.83
Cola + Primary enamel	383.8(±18.5)	115.2(±16.7)	69.01
Sprite + Permanent enamel	374.7(±17.6)	103(±15.3)	72.86
Sprite + Primary enamel	381.6(±19.7)	118.4(±21.6)	69.44
M. water + Permanent enamel	406.5(±16.3)	406(±9.7)	0.18
M. water + Primary enamel	413.7(±18.1)	412.5(±6.8)	0.25
Group 2 analysis			
Permanent enamel			
Cola + 2 mmol/L F.sulphate	407.6(±29.8)	212(±28.3)	46.87
Cola + 5 mmol/L F.sulphate	392(±22.9)	206(±13)	47.32
Sprite + 2 mmol/L F.sulphate	398(±29.8)	188.2(±28.3)	52.53
Sprite + 5 mmol/L F.sulphate	401(±24.6)	183.5(±16.4)	54.32
M. water + 2 mmol/L F.sulphate	408.2(±14.6)	404.1(±7.5)	0.93
M. water + 5 mmol/L F.sulphate	417.6(±11.5)	411.5(±11.1)	1.32
Primary enamel			
Cola + 2 mmol/L F.sulphate	413.1(±29.8)	216.2(±28.3)	46.68
Cola + 5 mmol/L F.sulphate	371(±18.6)	165.8(±19)	54.13
Sprite + 2 mmol/L F.sulphate	424(±29.8)	219(±28.3)	48.47
Sprite + 5 mmol/L F.sulphate	367.45(±25)	169.6(±21.6)	52.37
M. water + 2 mmol/L F.sulphate	419.6(±13.4)	416.1(±11.6)	0.78
M.water + 5 mmol/L F.sulphate	396.5(±18.2)	394.2(±10.1)	0.64

[Mean ± SD; n = 10];

SMH = Surface microhardness in Knoop hardness number

The surface microhardness (SMH) changes and the mean SMH loss percent from permanent and primary enamel following 3 cycles of 20 minute incubation of group-wise treatment with the acidic carbonated beverages and mineral water is shown. ($p<.05$)

amount of enamel lost compared to the clinical case[26]. First, *in vivo* the enamel surface will be covered by a protective pellicle and/or plaque layer[26]. In addition, the beverages contain significant amounts of organic acids which will stimulate salivary flow once they are introduced into the mouth. The resultant flushing and buffering effects of the increased salivary flow will help counteract the erosive effects of these products[26].

In the present study, the subjective assessment of macroscopic appearance of etching of the enamel surface was verified by measurement of the surface microhardness. Our results are similar to those of previous investigators that the most acidic drinks tend to show the greatest effects on enamel[21,28]. The present results showed a reduction of surface microhardness up to 56% and 61% with permanent teeth and primary teeth ($p<.0005$) respectively for the 5-min incubation period cycle (Table 3) and a further decrease by 72% and 70% in permanent teeth and primary teeth ($p<.0005$) respectively in the 20 minute incubation cycles (Table 4). This study is in concurrence with the findings of Seow WK and Thong KM[29], who also noticed a reduction of surface micro hardness of enamel surfaces when treated with acidic beverages.

The possible adverse effects of iron supplementation, such as teeth staining, possible modification of the taste of the beverage, as well as toxicity, must be taken into account when beverages are intended to be supplemented with this ion. Insufficient research on the effects of Fe ions at concentrations below 10mmol/L on enamel and on contemplating over factors such as toxicity and un-altered/acceptable tastes, this study incorporated much lower iron concentrations including 2 and 5mmol/L of the ferrous sulphate. Goldhaber studied the tolerable upper intake level for trace elements, proving that iron intake of up to 45mg/day posed no risk of adverse health effects[30]. WHO[31] has calculated a provisional maximum tolerable daily intake (PMDTI) of 56 mg/day (0.8 mg/kg/day), although lower levels have been defined in certain countries.

Supplementation of 2 and 5mmol/L $FeSO_4.7H_2O$ to the acidic carbonated beverages showed a definite calcium and phosphate release from enamel and surface microhardness reduction, the values that were statistically significant when compared to Group 1. This study however proved contradictory to the findings of a recent study that showed 1mM Fe ion supplementation on Sprite Zero in an *in vitro* situation to have no beneficial effect on dental erosion[32].

In the light of mechanisms considered for the reaction of iron with the dental enamel, it was shown that a barrier of ferric phosphate was formed which would reduce the contact of the soft drink with the enamel in the subsequent acid challenges and in turn diminish wear. Also, the indentor of the microhardness tester may have entered in contact not only with apatite (of the enamel), but also with the formed ferric phosphate layer thus reducing the loss of SMH observed when iron was supplemented[13].

When the 2 and 5mmol/L Ferrous sulphate were compared, no statistical significance was observed with respect to the surface micro hardness reduction in both the incubation periods, implying that lowering the concentration below 5mmol/L produced a similar effect (Table 3, 4). Hence, supplementation of acidic carbonated beverages with lower iron concentrations proved beneficial in reducing mineral loss and preserving surface microhardness of human enamel specimens. However, in long erosive challenges, spectrophotometry and surface hardness tests may not serve as sufficient tools to study dental erosion and thus further research should be directed towards measuring enamel loss in similar clinical situations by incorporating profilometry.

CONCLUSION

The supplementation of 2 and 5mmol/L Ferrous Sulphate in acidic carbonated beverages showed similar beneficial properties in reducing mineral loss and preserving surface microhardness of human enamel. Hence, considering the safety margin of iron supplementation in foods and beverages, lower concentrations of iron ($2mmolL^{-1}$) in the researched acidic carbonated beverages would seem to be a plausible alternative to reduce tooth mineral loss.

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