

Histological and microhardness evaluation of early artificial carious lesions in human and bovine enamel: *in vitro* study

Avaliação histológica e de microdureza de lesões incipientes de cárie em esmalte humano e bovino: estudo *in vitro*

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ABSTRACT

Objective: Early carious lesions in bovine and human enamel developed *in vitro* using a pH cycling regimen were compared. **Material and Methods:** Fifteen central bovine incisors and fifteen recently extracted human third molars were randomly divided into two groups: ten for the cross-sectional microhardness test (MT) and five for polarized light microscopy (PLM) analysis. Enamel blocks measuring 5 x 5 mm were made from the buccal face of the teeth. The blocks used for the MT were sliced into two halves: "A" and "B". "A" slices were embedded in acrylic resin, with the face of the dentin-enamel junction left exposed for the MT prior to pH cycling. "B" slices and whole blocks were coated with acid-resistant varnish, except a 3 x 3 mm central window, and submitted to the pH cycling regimen (demineralizing solution for 3 h and remineralizing solution for 21 h) over five consecutive days. The "B" slices were then submitted to the MT and the whole blocks were processed for the PLM study. **Results:** The PLM analysis revealed shallow, extensive lesions in the bovine enamel, hardly showing the superficial, dark and translucent zones, as well as deep cavity lesions in the human enamel, with the body of the lesion and the dark zone evident. The MT revealed a significant decrease in microhardness in the superficial levels of the bovine enamel caries and at all depth levels of the human enamel caries. **Conclusion:** The pH cycling regimen adopted led to the development of deeper and more demineralized carious lesions in human enamel than bovine enamel.

KEYWORDS

Dental caries; Dental enamel; Microhardness tests; Polarization microscopy.

RESUMO

Objetivo: Comparar lesões incipientes de cárie em esmaltes humano e bovino desenvolvidas *in vitro* através de ciclagem de pH. **Material e Métodos:** Quinze incisivos centrais bovinos e quinze terceiros molares humanos inclusos recém-extraídos foram aleatoriamente divididos em dois grupos: dez para o teste de microdureza transversal (MT) e cinco para a análise por microscopia de luz polarizada (MLP). Blocos de esmalte com dimensões de 5 x 5 mm foram confeccionados a partir da face vestibular dos dentes. Os blocos utilizados para MT, a qual foi determinada em cinco níveis de profundidade, foram seccionados em duas metades, designadas por "A" e "B". As fatias "A" foram submetidas ao teste de MT previamente à ciclagem de pH. Já as fatias "B" e os blocos de esmalte foram recobertos por verniz ácido-resistente, exceto por uma janela de 3 x 3 mm, e então submetidos à ciclagem de pH (soluções desmineralizadora por 3 h e remineralizadora por 21 h) por 5 dias consecutivos. As fatias "B" foram então submetidas a MT e os blocos foram processados para a MLP. **Resultados:** A análise por MLP revelou lesões rasas e extensas no esmalte bovino, com zonas superficial, escura e translúcida pouco visíveis; no esmalte humano, as lesões apresentaram-se profundas e cavitadas, com o corpo da lesão e a zona escura evidentes. A MT revelou significativa diminuição na microdureza nos níveis superficiais do esmalte bovino e em todas as profundidades avaliadas do esmalte humano após a ciclagem de pH. **Conclusão:** A ciclagem de pH levou ao desenvolvimento de lesões de cárie mais profundas e com maior desmineralização no esmalte humano em comparação ao esmalte bovino.

PALAVRAS-CHAVE

Cárie dental; Esmalte dental; Teste de microdureza; Microscopia de luz polarizada.

INTRODUCTION

The crystals of human enamel are densely packed and arranged in rods extending from the dentin to the enamel surface. Intact enamel contains up to 4% water by weight and up to 11% water by volume [1], forming a hydration shell around each apatite crystal. The remaining water mainly fills the pores between the rods as well as the minute pores within the rods. These pores form diffusion pathways into the enamel. Although bovine enamel crystals are larger and the prism diameters are smaller (with the considerable presence of interprismatic substance) in comparison to human enamel, these differences are not considered significant by some researchers [2-4].

Water in the intercrystalline spaces acts as a pathway for acid dissemination. When the pH value falls below the critical level, the mineral phase of enamel begins to dissolve and diffuses into the environment. In the early stages of enamel dental caries, this tissue undergoes mineral loss and/or changes in chemical composition as some ions, such as calcium and phosphate, bind to the tooth structure during the remineralization process, making the surface more resistant to demineralization [5].

The demineralization of human enamel in early carious lesions leads to more superficial mineral deposition than inner sub-surface mineral deposition. Therefore, the superficial layer remains relatively intact due to the precipitation of calcium, phosphate and fluoride released during the dissolution of crystals and the subsurface layer becomes soft. These characteristics of early human carious lesions are easily observed under polarized light microscopy (PLM), as reported by Silverstone (1973) [1]. However, it has been demonstrated that, although considerable initial surface demineralization occurs in early bovine enamel lesions, the inner remineralization is greater than that which occurs in human enamel; thus, the lesions tend to be less demineralized, shallower and with a thin or absent superficial layer

[6]. Furthermore, cattle have more balanced diet and shorter life span, which minimizes environmental influences in comparison to humans and leads to a certain homogeneity of the samples used in studies [6].

The microhardness test indirectly allows evaluating the initial stages of demineralization and remineralization of tooth enamel [7-9]. This test is also employed to compare the hardness of the substrate before and after treatments. Cross-sectional microhardness tests are used to evaluate specific changes in mineral content at different depths in carious lesions [10]. The literature reports a correlation between the cross-sectional microhardness of enamel and the content of calcium ions [11,2]. Sound bovine enamel exhibits lesser hardness than sound human enamel [13].

As bovine enamel has been used to replace human enamel in caries research due to the difficulty in obtaining human teeth under ideal ethical conditions [3, 14, 15], further studies are necessary to gain a better understanding of the characteristics of bovine enamel carious lesions.

The aim of the present study was to perform histological analyses and cross-sectional microhardness tests to compare early carious lesions developed *in vitro* using a pH cycling regimen on bovine and human enamel.

MATERIAL AND METHODS

This study received approval from the Research Ethics Committee of the School of Dentistry, University of São Paulo (protocol number 24/2009).

Fifteen extracted bovine incisors and fifteen recently extracted human non-erupted third molars with no visible enamel defects were stored in distilled water at 4°C. The superficial enamel on the buccal surface was polished on a rotatory polisher (Ecomet 4, Buehler, Lake Bluff, USA) with abrasive paper (grit # 180, 240, 320 and 400) to remove surface irregularities. After the removal of the roots, the crowns were divided in the mesiodistal direction. The lingual

slices were removed and the buccal slices were sectioned under water cooling using a precision cutting machine (Labcut 1010, Extec Corp. Enfield, USA) to obtain specimens measuring 5 x 5 mm.

The specimens used for the microhardness tests (10 bovine and 10 human) were longitudinally sectioned in the buccolingual direction for the obtainment of two halves (A and B). The A fragments were used for microhardness tests prior to pH cycling. For such, the specimens were embedded in acrylic resin with the dentin-enamel junction left exposed, which was polished with abrasive paper (grit # 600 and 1200). After the final stage of polishing, the test specimens were sonicated for five min to remove any residue. The B fragments were used for microhardness tests after pH cycling. Moreover, whole blocks (5 bovine and 5 human) were processed for the PLM analysis.

For pH cycling, steel wires were attached to each specimen and the surfaces were coated with acid-resistant varnish, except on areas measuring 3 x 3 mm to be exposed to the pH cycling solutions. The specimen/wire units were immersed in individual tubes containing distilled water until use. The specimens were then placed in a demineralizing solution (2.0 mM calcium and 2.0 mM phosphate in 0.075 M acetate buffer, pH 4.3) at 37 °C for 3 h (60 ml per specimen), followed by a remineralizing solution (1.5 mM calcium, 0.9 mM phosphate and 150 mM of KCl in 0.1 M Tris buffer, pH 7.0) for 21 h (30 ml per specimen). The procedure was repeated daily. After five days, the specimens were removed from the solutions and placed in distilled water.

For the cross-sectional microhardness analysis, the enamel cross-sections (at the middle of the lesion in a buccolingual direction) were processed by placing three indentations spaced 20 μ m apart on five lines starting from the external enamel surface along three parallel rows using a Shimadzu HMV-2000 (Shimadzu Corp, Kyoto, Japan) microhardness tester with a Knoop diamond indenter (25 g/10 s).

For the histological analysis, specimens were embedded in epoxy resin (Redelease, São Paulo, Brazil) and sectioned (Isomet 1000,

Buehler LTDA, Illinois, USA) in the buccolingual direction, resulting in slices measuring 300 μ m in thickness. Three sections were randomly chosen from the central area of the lesions and hand ground to a thickness of 80 to 150 μ m using sandpaper (human specimens were thicker than bovine specimens due to difficulties in hand ground). Each specimen was examined under a light microscope with polarization lens, imbibed in water (Zeiss, Deutschland).

RESULTS

On the microscopic images, early caries in bovine enamel appeared as restricted rectangular areas on the surface of the specimen. Although the large thickness of some specimens do not permit a real polarization of the light, it was possible to distinguish the caries layers. The inner portions of the lesions were marked by a dark line, which divided the healthy enamel from the demineralized enamel and likely represented the dark zone. Another zone was found below the first, in which the coloration differed from that of healthy enamel, but it was lighter than that of the dark zone and likely represented the translucent zone. The body of the lesion appeared as a clear zone and was not always detected. The demineralized area generally appeared as a strip with coloration differing from that of healthy enamel, with no clear distinction of the different carious layers (Figure 1 - A).

The histological images of the human enamel lesions appeared as cavitated lesions both macroscopically and microscopically. This may have occurred due to the processing of the specimens, nonetheless indicates a high degree of demineralization. The images showed extensive, deep lesions, reaching nearly half the thickness of the enamel, with the complete loss of the superficial zone and body zone of the lesion. Although the large thickness of some specimens do not permit a true polarization of the light, it was possible to distinguish the caries layers. A thick, dark layer of demineralized enamel was observed, likely representing the dark zone, under which a fine line was found, likely representing the translucent zone (Figure 1 - B).

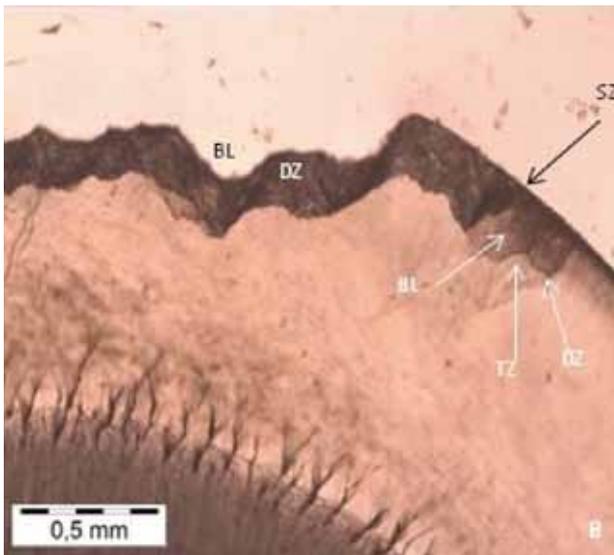
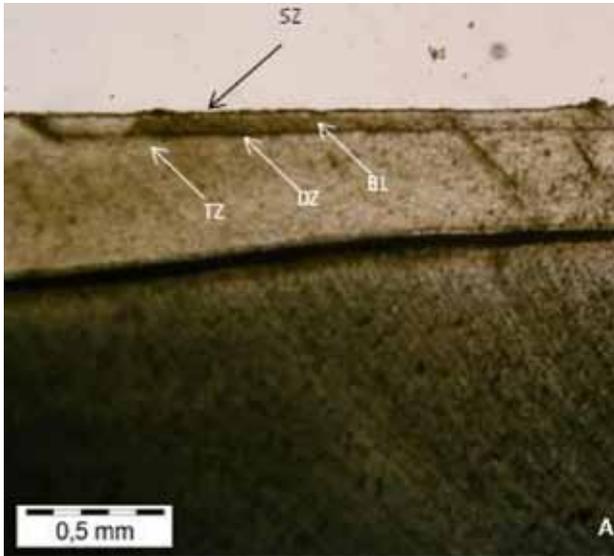


Figure 1- A – Carious lesion in bovine enamel examined under polarized light microscope (magnification: 40x); B – Carious lesion in human enamel examined under polarized light microscope (magnification: 40x). SZ=superficial zone. BL= body of lesion. DZ = dark zone. TZ = translucent zone.

The histological images of the human The analysis of the cross-section revealed a significant reduction in microhardness in the superficial levels of the bovine enamel (Table 1). In contrast, a significant reduction in microhardness was found at all depths in the human enamel (Table 2).

Table 1- Cross-section microhardness in non-demineralized and demineralized bovine enamel at five depth levels (Wilcoxon Signed-Rank Test)

Pairs of different depth levels	Mean Value (KHN)	Standard Error	Significance (P)
A_D1	267.41	47.96	0.009
B_D1	140.27	15.53	
A_D2	267.94	40.98	0.017
B_D2	162.32	33.22	
A_D3	287.40	41.92	0.059
B_D3	210.87	43.02	
A_D4	290.30	42.40	0.059
B_D4	212.44	41.28	
A_D5	293.20	42.82	0.047
B_D5	219.36	42.75	

D1, D2, D3, D4, D5: Depth levels of enamel lesions from surface to dentin/enamel junction.
A: sound enamel / B: demineralized enamel.

Table 2- Cross-section microhardness in DE-RE human enamel at 5 depth levels (Wilcoxon Signed-Rank Test)

Pairs of different depth levels	Mean Value (KHN)	Standard Error	Significance (P)
A_D1	340.83	25.13	0.005
B_D1	256.83	26.19	
A_D2	349.73	19.36	0.013
B_D2	308.40	13.62	
A_D3	360.67	25.93	25.93
B_D3	306.13	19.89	
A_D4	367.77	25.15	0.013
B_D4	312.53	18.87	
A_D5	362.33	22.49	0.017
B_D5	315.67	18.34	

D1, D2, D3, D4, D5: Depth levels of enamel lesions from surface to dentin/enamel junction.
A: sound enamel / B: demineralized enamel.

DISCUSSION

The pH cycling regimen simulates the development of carious lesions *in vitro*, alternating pH solutions, which is similar to what occurs in the demineralizing and remineralizing processes in the oral environment [16]. In the present study, pH cycling led to the development of early carious lesions in bovine enamel, using the same methodology described by Featherstone (1996) [17] and modified by Gama-Teixeira (2007) [18] and Argenta, Tabchoury and Cury (2003) [9] for human teeth. This result is similar to findings reported in previous studies [9,11,16,19]. Although a true polarization microscopy was not possible in some specimens the images showed that demineralization zones were observed on all the exposed bovine enamel surfaces. However, these lesions were not as deep as those reported in studies on human teeth [9, 18]. In the present study, the carious lesions on human enamel exhibited deep demineralization zones as well as the complete loss of surface tissue; the body of the lesions was evident and the superficial layer may have been lost during the specimen preparation due to the high degree of demineralization. These findings differ from those described by Silverstone (1973) [1] as well as from the lesions found on bovine enamel specimens.

According to Lynch and Ten Cate (2006) [20], remineralization is proportional to initial mineral loss during demineralization. As the characteristics of bovine enamel favor demineralization due to the greater number of crystals, smaller prisms and greater presence of interprismatic substance, remineralization occurs proportionally, forming a more evident translucent zone. Studies also suggest that more porous lesions may be more easily remineralized than less porous lesions [21]. Therefore, the body of the lesion may be thinner in bovine early enamel lesions than in human enamel lesions.

The cross-sectional microhardness analysis indirectly indicates the degree and depth of demineralization and has been widely used for the analysis of human teeth [7-9]. The results

of the cross-sectional microhardness analysis in the present study, however, revealed high side deviation. The same high side deviation values can be observed on both sound and demineralized human enamel. This fact points to the considerable variability in microhardness in sound bovine and human enamel, which should be considered when these tissues are compared in any study. In the bovine enamel specimens, the analysis revealed significant differences in microhardness between sound and carious enamel in the two most superficial levels, confirming the findings of the histological analysis, which revealed a very thin zone of demineralization. In the human enamel specimens, significant differences in microhardness were found between sound and carious enamel at all five depth levels analyzed, confirming the histological analysis, which revealed a thick zone of demineralization with superficial tissue loss (cavitated lesions). These findings are in agreement with those reported in previous studies [6-9, 13, 20].

CONCLUSION

The pH cycling protocol used in the present study led to the development of deeper and more demineralized carious lesions in human enamel in comparison to bovine enamel.

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