

# Erosion-like lesions progression in human and bovine enamel

## *Progressão de lesões erosivas em substrato humano e bovino*

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## INTRODUCTION

There have been substantial advances in understanding the pathophysiology of

## ABSTRACT

Although on dental erosion studies bovine enamel has been considered a feasible option to human enamel, time dependence related to the erosive challenge remains uncertain. Thus, in this study the following null hypothesis was tested: 1) independent of the number of erosive challenges, no microhardness (SMH) differences exist between human and bovine enamel and 2) the behavior of bovine substrate along the erosive challenges do not differ from the human substrate. Human and bovine enamel slabs were embedded in epoxy resin and serially polished and flattened. Initial SMH tests were measured. The erosive challenge consists of an immersion of the specimens in continuously stirred orange juice (20ml) for 5 minutes at room temperature twice a day, for 5 days. Between the immersions the specimens were washed with deionized water and stored in artificial saliva (20ml). SMH tests were daily measured at the end of 2,4,6,8 and 10 challenges. Data were analyzed by two-way ANOVA as a split-plot design in time and Tukey's test. Regression analyses were used to model the SMH of human and bovine enamel over time. The first null hypothesis was rejected. Regression analysis exhibits a quadratic polynomial reduction of SMH in function of the number of erosive challenges for both substrates, revealing the admission of the second null hypothesis. Bovine substrate showed the most susceptible substrate to erosive challenge, but after several days of erosive challenge this difference disappears. Thus, the use of bovine tooth instead of human tooth in studies of erosive lesions formation seems to be feasible.

**Key words:** Erosion; microhardness; human enamel; bovine enamel.

## RESUMO

Embora em estudos de erosão, o emprego do esmalte bovino seja considerado uma opção viável ao esmalte humano, a comparação do comportamento da indução das lesões erosivas nos diferentes substratos em relação à progressão/perda mineral permanece incerto. O objetivo deste estudo foi definir *in vitro*, a partir de um protocolo de indução de desgaste corrosivo, o tempo para formação de lesões cujo padrão, avaliado através de microdureza Knoop, seja semelhante em substratos bovinos e humanos. Fragmentos esmalte humano e bovino foram incluídos em resina epóxica, planificados, polidos. Um protocolo para o desenvolvimento de lesões erosivas foi instituído e cada ciclo erosivo consistiu da imersão dos espécimes em 20 ml de suco de laranja, em temperatura ambiente, por 5 minutos, sob agitação, duas vezes ao dia, durante 5 dias. No período entre as exposições e durante a noite, os espécimes foram mantidos em 20 ml de saliva artificial. Após o desafio erosivo leituras de microdureza foram efetuadas para avaliar a perda de conteúdo mineral. A análise de variância mostrou efeito significativo na interação substrato - tempo (p,0096). Ao longo dos episódios erosivos, os substratos dentais - humano e bovino - apresentaram um comportamento semelhante. A microdureza para ambos os substratos variou de acordo com uma função polinomial de segundo grau onde apesar de inicialmente não existir diferença entre os valores de microdureza dos substratos, o esmalte bovino se mostrou mais susceptível aos episódios erosivos; porém com o aumento do número de desafios, a diferença entre os substratos desapareceu.

**Palavras-chave:** Erosão dental; microdureza; esmalte humano; esmalte bovino

dental erosion, especially through controlled *in vitro* studies. Regarding the tooth wear, the use of *in vitro* studies showed some advantages, like the high level of scientific

control, ethical aspects related to certain experiments, reduction in experimental variability, the use of highly sensitive assessment methods, and the possibility of separate the components of tooth wear and investigate them separately.

Erosion-like lesions have been produced through different models, using both human<sup>1-5</sup> and bovine teeth<sup>1,5,6,7</sup>. Bovine substrate is widely used in dental research and presents similarities with human enamel such as the chemical composition<sup>8</sup>, crystal orientations<sup>9</sup> and microhardness<sup>10,11</sup>. Bovine teeth have some advantages in relation to the ethical aspects, being easier to obtain and manipulate than human teeth<sup>10</sup>. In fact, ethics committees are encouraging their use as an alternative for human substrate<sup>12</sup>. However, bovine enamel cannot be replaced for human enamel without validation<sup>8</sup>.

Although bovine enamel has been considered a feasible option to human enamel, few studies have compared artificial erosion lesions progression in both substrates<sup>1,5,6</sup>. The aim of this study was to evaluate whether an erosive challenge protocol that simulates acidic drinks intake is able to induce similar erosion-like lesions in human and bovine enamel.

## MATERIAL AND METHODS

### *Experimental design*

This study was designed as a randomized complete block with 15 replications and a split-plot factorial arrangement. The factors under study were: *dental substrate* at two levels (bovine and human enamel) and *number of erosive challenges*, at six levels, repeatedly measured on the same specimen, at different times (T0, T1, T2, T3, T4, and T5). The levels of the factor *dental substrate* were assigned to the main plot and the factor *number of erosive challenges* were assigned to the subplots. The following null hypothesis were tested: 1) independent of the number of erosive challenges, no microhardness (SMH) differences exist between human and bovine enamel and 2) the behavior of bovine substrate along the erosive challenges do not differ from human substrate. The response variable was surface microhardness (SMH).

### *Preparation of specimens*

Sound third human molars and bovine

incisors were cleaned to remove tissue remnants and stored in a 0.1% thymol solution, at 4°C. Each tooth was sectioned using a low-speed water-cooled diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA) in order to obtain enamel slabs (3 x 2 x 2 mm) that were inspected for defects under a stereomicroscope at 40x magnification and discarded if pitted or cracked. The preselected slabs were embedded in epoxy resin (Epoxicure Resin, Buehler) with enamel surface facing up and flattened in a water-cooled polishing machine (Beta Grinder-Polisher, Buehler) using 400, 600 and 1200-grit Al<sub>2</sub>O<sub>3</sub> papers, and polished on cloths with a 0.3 µm alumina suspension (Alpha and Gamma Micropolish, Buehler, Lake Bluff, IL, USA). Specimens were ultrasonically cleaned in deionized water (T1440D, Odontobrás, Ribeirão Preto, SP, Brazil) for 10 minutes and stored at 37° C in 100% relative humidity.

### *Baseline microhardness measurements*

Specimens of human and bovine enamel were tested for surface Knoop microhardness (HMV-2, Shimadzu, Kyoto, Japan), under an load of 25 g, applied for 30 s. Indentations were performed in each specimen at 150, 500 and 1500 µm from superior margin, and at 100 µm to the lateral margin. The microhardness was measured with the aid of dedicated software (New Age, Software Cams, South Ampton, PA, USA) and the average of surface microhardness (SMH) values was calculated. Specimens with microhardness values 20% above or 20% below the average value were discarded.

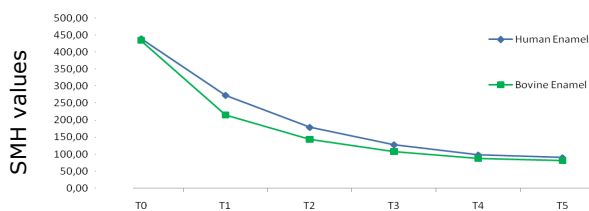
### *Erosive Challenges*

The erosive challenge was based on that proposed by Amaechi et al, (1999). Briefly, the protocol consisted of immersing the specimens in 20 mL of pure ready to drink orange juice (pH = 3.74) (Fazenda Bela Vista, Tapiratiba, SP, Brazil) in an erlenmeyer flask, which was then placed in an orbital shaker (CT155, Cientec, Piracicaba, SP, Brazil), with stirring velocity of 100 rpm for 5 minutes, at the room temperature (25°C), twice daily, for 5 days. From one immersion to the other, the specimens were washed with deionized water and stored in 20 mL of the artificial saliva (pH of 6.75), described by McKnight-

Hanes and Whitford<sup>13</sup> and modified by Amaechi et al.,<sup>2</sup>, at 37°C until the following erosive episode. Saliva was daily changed and the orange juice at each immersion.

*Microhardness analysis along the erosive challenges*

Surface microhardness measurements (SMH) were performed following the lesion progression at 250 µm left from the measurements made at the post-indentation. SMH analyses were assessed under the same load and time described above for the baseline measurements. The analysis was carried out at the end of each day, after the two daily challenges, and to clarify this, six points in time were set (T0, T1, T2, T3, T4, T5) (Figure 1). The times are in equivalency with levels of the factor *number of challenges*, thus, T0 means the initial condition, while the time T5 refers to the end of the five day, which means, 10 erosive challenges.



**Figure 1:** Erosion-like lesions behavior in relation to microhardness changes over time for human and bovine enamel.

*Statistical Analysis*

Data analysis was carried out using Statgraphics Centurion XV (Manugistics, Rockville, MD, USA) at a significance level of  $\alpha = 0.05$ . After assumptions of normality and homogeneity of variances had been verified, SMH data were analyzed by two-way ANOVA as a split-plot design. Post-hoc pair wise multiple comparisons were performed using the Tukey’s test applied at each time (T0, T1, T2, T3, T4 and T5) to identify differences in microhardness values between human and bovine substrate (L.S.D - 22.44). Regression analyses were used to model the SMH of human and bovine enamel, individually, over time.

**RESULTS**

The first null hypothesis was rejected, since human and bovine substrate showed

differences regarding the SMH measurements. However, the second null hypothesis was accepted, regression analysis exhibits a quadratic polynomial reduction of SMH in function of the number of erosive challenges for both substrates. A two-way ANOVA according to a split-plot design revealed significant interaction between *dental substrate* and *number of erosive challenges* ( $p = 0.0096$ ). At the times T0, T3, T4 and T5, SMH values of human and bovine enamel did not differ from each other, as shown in Table 1. After 2 and 4 erosive challenges (T1 and T2), human enamel showed higher SMH than the bovine substrate. The SMH values for both human and bovine enamel decreased SMH, according to the following models:

For human enamel:  
 $SMH = 429,375 - 159,839 * Time + 18,7089 * Time^2$  ( $r^2 = 90,3\%$ )  
 For bovine enamel:  
 $SMH = 409,545 - 175,832 * Time + 22,6783 * Time^2$  ( $r^2 = 92,0\%$ )

**Table 1 –** Post-hoc pair wise multiple comparisons performed by Tukey’s test at each time of the induced lesions for both substrates<sup>1</sup>.

	T0	T1	T2	T3	T4	T5
Human	439.2 A	273.1 A	178.6 A	128.1 A	98.3 A	90.1 A
	(37.8)	(63.4)	(50.2)	(26.7)	(24.0)	(20.4)
Bovine	433.7 A	215.5 B	142.7 B	107.4 A	86.7 A	80.9 A
	(34.9)	(42.6)	(26.1)	(17.6)	(17.2)	(17.4)

\*Capital letter showed statistical difference ( $\alpha = 0,05$ ) between the experimental groups in column. I.s.d. = 22,44.

**DISCUSSION**

Erosive lesions involve enamel apatite crystals dissolution, which is associated with a surface softening caused by the frequent contact with acids. This process might weaken the enamel structure, and when the softened enamel is not re-hardened, they become more vulnerable to further dissolutions or physical insult, leading to an erosion lesion formation<sup>14,15,16</sup>.

<sup>1</sup> Equivalency with levels of the factor *number of challenges* T0 = 0 pre challenge condition; T1 = after 2 challenges, or even, at the end of first day; T2 = after 4 challenges or even, at the end of second day; T3 = after 6 challenges, or even, at the end of third day; T4 = after 8 challenges or even, at the end of fourth day; T5 = after 10 challenges, or even, at the end of fifth day.

Orange juices may cause an appreciable level of erosion on enamel substrate<sup>2,6</sup> and to simulate the acidic drinks intake, the model used to induce erosion like lesions was based on ready to drink orange juices. To induce erosion-like lesions, the chosen method was originally proposed for microradiographic assessments<sup>2</sup>, and as the study showed, the prolonged intermittent exposure of enamel slabs to orange juice increases progressively the depth of erosion lesion and the loss of mineral content, measured by microradiography<sup>2</sup>. However, although microradiography and even profilometry are accepted methods to assess erosion lesions<sup>11</sup>, it was not included in this study.

Since this study was designed to follow the initial stages of erosive lesions formation, preliminary tests showed that the protocol proposed by Amaechi et al.,<sup>2</sup> was aggressive, thus, instead of six times a day for 24 days, the protocol was modified for twice daily challenges for five days. Regarding the response variable, as erosion has been considered a surface phenomenon<sup>17</sup>, to adapt the methodology, following the study objective, surface microhardness was chosen as evaluation method.

As a simple non-destructive method to assess the early stages of enamel erosion, indentation techniques have been extensively used to investigate enamel erosion by measuring the hardness of the enamel surface<sup>7,11,18</sup>. Surface Knoop microhardness was chosen, since this method allowed measurements along the time in the same specimen<sup>19</sup>, an aspect taken into account regarding the hypothesis initially proposed.

The SMH values throughout the elapsing process of lesions formation for both, human and bovine enamel showed a similar behavior (Figure 1). The adopted split plot design allow to obtain the equation of the fitted model for human and bovine enamel, and for both substrates the  $r^2$  observed has a value higher than 90%, which means that the data fitted tightly to the estimated model, and for both substrates, quadratic polynomial equations were obtained modeling the decrease of SMH values over time.

Considering that the microhardness of both substrates was assumed to be the same, previously to erosive challenge (T0) (Table 1), the SMH values of human and bovine enamel were similar; in fact, it has

been showed that the mineral contents of both substrates are not significantly different<sup>1,5,8,10,20</sup>.

After the times T1 and T2 (Table 1), bovine enamel showed lower SMH values when compared to human substrate. Considering that bovine enamel had a faster erosive lesion progression than in human permanent enamel, our results corroborates with Amaechi et al.,<sup>2</sup> and Attin et al.,<sup>5</sup>, and, this finding may be related to the higher porosity of bovine enamel<sup>21</sup>. Microscopically, human and bovine enamel exhibit similar crystals orientations, although bovine teeth exhibit a wider inter-prismatic region<sup>9</sup>, with bigger crystals than human enamel,<sup>22</sup> an structural aspect that may enhance the acid diffusion promoted by the erosive challenge acting on the tissue mechanical resistance, weakening the enamel structure, leading to lower microhardness values. Thus, the SMH values obtained in the times T1 and T2 of our study reinforces this suggestion, since microhardness indentations is a function of the mechanical properties of the material<sup>11</sup>.

Nevertheless, as the erosive process continues, in the times T3, T4 and T5, the difference between both substrates was no longer observed. The increase of erosive challenges along the time seems to be decisive to induce similar lesions in both human and bovine enamel. Despite the faster demineralization<sup>23</sup> of bovine enamel due to its porosity<sup>21</sup>; with the high dissolution of apatite crystals caused by the acidic contact<sup>1</sup>, when hardness or even mineral content are reduced, the differences seems to be irrelevant. These results suggest that in the dependence of erosive challenges, the bovine enamel represents a viable alternative to human substrate.

## CONCLUSION

Bovine substrate showed the most susceptible substrate to erosive challenge, but after several days of erosive challenge this difference disappears. On erosion research, the use of bovine tooth instead of human tooth seems to be a feasible alternative.

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