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# Development of caries-like lesions in human and bovine dentin compared to natural caries

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## Resumo

Dada a complexidade, aspectos éticos e custos envolvidos na condução de experimentos clínicos relacionados a cárie dental, estudos in vitro têm sido uma alternativa às pesquisas clínicas. O objetivo deste estudo foi avaliar o desenvolvimento de lesões artificiais de cárie em dentina humana e bovina, comparado à lesões de cárie natural em dentina humana (CT). Quinze fragmentos de dentina humana naturalmente cariada, 45 fragmentos de dentina humana (H) e 45 fragmentos de dentina bovina (B) foram planificados e polidos. Os espécimes dos grupos H e B foram então submetidos a três protocolos de ciclos de pH: 8 (H8 e B8), 12 (H12 e B12) e 16 ciclos (H16 e B16). Após o desafio cariogênico, valores de microdureza Knoop (KHN) foram obtidos a 30, 60, 90, 120 e 150 µm da superfície das lesões. A Análise de Variância mostrou efeito significativo da interação ciclo-profundidade. O teste *t*-Student foi empregado para comparar as médias de KHN do substrato naturalmente cariado (CT) em relação a cada desafio cariogênico desenvolvido nos grupos H e B, em cada profundidade. Os modelos de ciclos de pH que mais se aproximaram em termos de microdureza das lesões de cárie natural foram o grupo B8, para dentina bovina e o grupo H16, para dentina humana. Dentro das condições do presente estudo, pode-se concluir que a utilização tanto de substrato humano quanto bovino pode ser considerada uma alternativa viável para o desenvolvimento de lesões de cárie artificial.

**Palavras-chave:** Microdureza; dentina humana; dentina bovina; cárie dental.

## Abstract

Due to the complexity, ethical aspects and high costs involving clinical experiments on dental caries, in vitro studies have been considered as an alternative option to clinical researches. The purpose of this study was to compare artificial caries-like lesions in human and bovine dentin to natural caries in human dentin (CT). Fifteen specimens of human dentin with natural caries, 45 specimens of human dentin (H), and 45 specimens of bovine dentin (B) were flattened and polished. The specimens from groups H and B were submitted to three different protocols of pH cycles: 8 (H8 and B8), 12 (H12 and B12), and 16 cycles (H16 and B16). Each cycle consisted of immersion of the specimens for 6 hours in a demineralising solution and for 18 hours in a remineralising solution. After the cariogenic challenge, the Knoop microhardness (KHN) was determined at 30, 60, 90, 120 and 150 µm from the lesion surface. The Analysis of Variance showed a significant effect of the interaction cycle-depth. The Student's *t*-test was employed to compare the mean values of KHN from the substrate with natural caries to the values of KHN of each cycle of the experimental groups (H or B), at each depth. The pH-cycling models closest to natural caries were group B8, for bovine dentin and group H16, for human dentin. Within the conditions of the present study, it can be concluded that the use of both human and bovine substrates can be considered a viable alternative to the development of artificial caries lesions.

**Keywords:** Microhardness; human dentin; bovine dentin; dental caries.

## INTRODUCTION

The mechanism of dental caries development has been widely investigated in the last decades,[1-11] making the caries process well-understood.[10] Although the formation of caries lesions seems to be simple in concept, it is in fact a complex and very detailed process.[10]

Several models have been developed to simulate the caries process in human[1-3,7] and bovine enamel.[5,7,9,12,13] Bovine enamel represents a feasible option in the caries research, since it is easier both to obtain and to manipulate.[14,15] In addition, it presents a more uniform chemical composition, which might allow a lower variation in the experiment response of cariogenic and anticariogenic treatments.[14]

There are still many details to be determined regarding the protocol of cariogenic challenge to be followed, in order to produce caries lesions closely resembling those that occur naturally, especially in dentin. Thus, several in vitro models have been proposed to induce caries-like lesions in dentin.[8,16,18,21,22] Human[16] and bovine dentin[8] have already been used to compare different models of in vitro root caries, suggesting the formation of lesions with similar features to those of natural caries. However, studies involving the comparison between caries-like lesions induced in vitro in human and bovine dentin and natural caries in human dentin have not been reported yet.

The aim of this study was to compare artificial caries lesions, induced in vitro in bovine or human dentin, to naturally-developed lesions in human dentin. The focus was to verify which in vitro protocol could produce caries-like lesions in bovine and human dentin that most closely resembled the natural dental caries.

## MATERIALS AND METHODS

### 1. *Ethical Aspects*

The present research was approved by the National Research Ethics Committee (CONEP), CAAE No. 0003.0.138.000-0.

### 2. *Experimental Design*

The experiment was carried out in two dental substrates, bovine and human dentin, which were compared to natural caries in human dentin. Forty-five specimens of human dentin (H) and 45 specimens of bovine dentin (B), were chosen at random, to be submitted to three different models of pH cycling (8, 12 and 16 cycles) (n = 15), in order to induce caries lesions formation. Fifteen specimens with natural caries lesions in human dentin (CT) were also used (n = 15) (Table 1). The response variable was Knoop microhardness (KHN).

### 3. *Specimens Preparation*

Recently-extracted bovine incisors, sound human molars, and human molars with occlusal caries lesions were cleaned to remove tissue remnants and stored in 10% formalin solution (pH 7.0).

The teeth were sectioned at the cement-enamel junction using a low-speed water-cooled diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA). The molars with natural caries lesions had their crows sectioned in the mesial-to-distal direction through the caries lesion, allowing carious dentin exposure. The human sound molars also had their crowns sectioned in mesial-to-distal direction. Dentin slabs were obtained from the buccal surface of bovine incisors and human sound molars. The enamel was grounded up to dentin exposure, by means of a water-cooled mechanical grinder (Struers A/S, Copenhagen, Denmark). He samples had the dentin surfaces serially polished with 400, 600 and 1200-grit Al2O3 abrasive papers, and 0.3 and 0.05 µm alumina polishing suspensions (Alpha and Gamma Micropolish, Buehler, Lake Bluff, IL, USA) on cloths. Specimens were ultrasonically cleaned in deionized water for 10 minutes,[13] inspected for defects under a stereomicroscope (Nikon 88286, Tokyo, Japan) at 40× magnification, and discarded if pitted or cracked. Dentin slabs were individually stored at 37 °C in 100% relative humidity.

### 4. *Specimens Selection*

Specimens of sound human and bovine dentin were tested for surface microhardness (HMV-2, Shimadzu, Kyoto, Japan) under 10 g load applied for 20 seconds.

Three indentations, spaced on 500 µm, were performed on the surface of each slab. Specimens with microhardness values 30% above or 30% below the mean value of all specimens were discarded. For natural dentin caries lesion selection, an aspect to be considered is related to the limitations regarding this substrate. Considering that it is very difficult to obtain natural caries in human molars with a standardized lesion condition, some criteria were taken into account to include lesions with similar

**Table 1.** Experimental groups according to the number of pH cycles

| **Control group (CT)** | **Nature of the caries lesion** | **n** |
|---|---|---|
| | Specimens with natural caries lesions | 15 |
| **Bovine groups (B)** | **Nature of the caries lesion** | **n** |
| B8 | Specimens submitted to 8 pH cycles | 15 |
| B12 | Specimens submitted to 12 pH cycles | 15 |
| B16 | Specimens submitted to 16 pH cycles | 15 |
| **Human groups (H)** | **Nature of the caries lesion** | **n** |
| H8 | Specimens submitted to 8 pH cycles | 15 |
| H12 | Specimens submitted to 12 pH cycles | 15 |
| H16 | Specimens submitted to 16 pH cycles | 15 |

characteristics, so as to perform a representative control group for dentin natural lesions.

Human molars with natural caries lesions (CT) were initially selected through visual inspection. The presence of active lesions was taken into consideration, as an extension of demineralisation along the enamel dentin junction, involving at least ¼ of the dentin lesion extension depth. For microhardness measurements, the used criteria were based on the hardness readings, measured at different depths, in order to sweep the entire lesion length.

## 5. *Cariogenic Challenge*

Human (H) and bovine (B) dentin slabs were isolated with wax, leaving an exposed area of 4.15 $mm^2$ of dentin, which remained in contact with the solutions during the induction of caries lesions. The pH cycling consisted of individual immersion of the samples in 10 mL of demineralising solution (1.4 mM Ca, 0.9 mM P, 0.05 M acetate buffer, pH 5.0) for 6 hours and 5 mL of remineralising solution (1.5 mM Ca, 0.9 mM P, 0.1 M Tris buffer, pH 7.0) for 18 hours.[24] The number of cycles carried out was 8, 12 and 16, according to the experimental group (Table 1). From one immersion to the other, the specimens were washed with deionized water.

## 6. *Embedding of Specimens in Resin*

At the end of the pH cycling, the specimens of human (H) and bovine (B) dentin were sectioned through the centre of the area exposed to the cariogenic challenge, exposing the subsurface lesion. They had the cross-sectioned surface embedded in polyester resin and polished with $Al_2O_3$ abrasive papers of decreasing abrasiveness (600 and 1200) and 0.3 and 0.05 µm alumina polishing suspensions on cloths, under water cooling. Afterwards, the specimens were washed with deionized water, immersed in deionized water, and sonicated for 10 minutes to clean the surface.[13]

## 7. *Cross-Sectional Microhardness Analysis*

The depth of artificial caries lesion was analyzed by means of cross-sectional microhardness. Measurements were obtained from the lesion surface at five depths (30, 60, 90, 120 and 150 µm), in triplicate, by using a static load of 10 g applied for 20 seconds to the specimens of human (H) and bovine (B) dentin with artificial caries lesions. The chosen depths were considered, as the obtained artificial caries lesions did not exceed the 150 µm in depth.

## 8. *Natural Caries Microhardness Analysis*

For natural caries lesions in dentin, the microhardness analysis was assessed under the same load and time described above for the experimental groups, as well as at the same depths. Five indentations were performed, in triplicate, from the outer portion of the dentin towards the pulp chamber. Samples with natural caries were considered as the control group (CT) (n = 15) (Table 1).

## 9. *Statistical Analysis*

Since the group with natural caries lesions (CT) could not be chosen at random, this restriction prevented the Analysis of Variance (ANOVA) for all experimental groups.[25] The Student's *t*-test was employed to compare the mean values of microhardness in each cycle of the experimental groups (H or B) to those of the control group (CT), at each depth (Table 2), and using the software STATA for Windows version 6.0 (StataCorp, College Station, TX, USA).

**Table 2.** Microhardness (KHN) values (mean ± sd; n = 15) for human and bovine dentin subjected to pH cycles compared to control group

| DEPTH (µm) | | 30 µm | | 60 µm | | 90 µm | | 120 µm | | 150 µm | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control group (CT) | | 5.96 | A | 7.94 | A | 11.36 | A | 14.86 | A | 25.05 | A |
| | | (2.88) | | (3.12) | | (6.60) | | (8.55) | | (13.49) | |
| **Experimental groups** | **Cycles** | | | | | | | | | | |
| Bovine dentin (B) | 8 | 4.03 | B | 6.32 | A | 8.32 | A | 11.48 | A | 34.90 | B |
| | | (0.80) | | (1.20) | | (0.78) | | (1.15) | | (5.81) | |
| | 12 | 2.60 | B | 3.42 | B | 4.24 | B | 6.61 | B | 12.19 | B |
| | | (0.51) | | (0.96) | | (0.47) | | (0.61) | | (2.22) | |
| | 16 | 2.26 | B | 3.14 | B | 6.36 | B | 10.33 | A | 14.46 | B |
| | | (0.26) | | (0.56) | | (1.38) | | (0.86) | | (1.39) | |
| Human dentin (H) | 8 | 2.86 | B | 6.63 | A | 15.01 | B | 21.81 | B | 23.12 | A |
| | | (0.63) | | (0.88) | | (1.32) | | (1.69) | | (1.09) | |
| | 12 | 2.59 | B | 7.04 | A | 12.71 | A | 21.16 | B | 24.78 | A |
| | | (0.50) | | (0.95) | | (0.94) | | (1.21) | | (0.98) | |
| | 16 | 2.52 | B | 6.82 | A | 9.38 | A | 14.98 | A | 25.63 | A |
| | | (0.52) | | (1.16) | | (1.25) | | (1.26) | | (1.16) | |

*The same capital letter shows statistical similarity between each experimental group and the control group (Student's *t*-test; $p < 0.05$).

## RESULTS

The mean values of microhardness of H and B experimental groups are illustrated in Figures 1 and 2, respectively. The Student's *t*-test showed that the bovine experimental groups presented lower microhardness values in comparison with the control group, at depths of 30 and 150 µm, except for group B8, which showed a higher microhardness value at 150 µm. At the depths of 60, 90 and 120 µm, there was statistical difference for groups B12 and B16, which presented lower microhardness in comparison with the control group, except at the depth of 120 µm with 16 cycles. Group B8 was similar to the control group at the depths of 60, 90 and 120 µm (Table 2).

The human experimental groups H8, H12 and H16 showed microhardness values which were lower than that of the control group at 30 µm. None of the human experimental groups presented difference at the depths of 60 and 150 µm, in comparison with the control group. Group H8 presented statistically higher microhardness compared to that of the control group at 90 and 120 µm, as well as group H12 at 120 µm (Table 2).

## DISCUSSION

The response variable used in the present study was the Knoop microhardness, a method of analysis usually employed in studies of progression and inhibition of caries lesions.[1-7,21-23] Other methods have also been used for the assessment of caries lesions, such as the polarized light microscopy,[8,15,16,24] which allows distinguishing sound and demineralized substrates by differences in the tissues birefringence,[15,16] and the microradiography,[6,9,17,19] a method employed to evaluate the mineral content in dental substrates.[6,9] The advantages of microhardness over the other methods are its lower cost and the simplicity of its technique.[26]

Microhardness reflects both the organic and inorganic content of dental substrates[6] and is still widely applied in dental caries studies.[12,13,16,21-23] However, when used in dentin, this method could be criticized due to the indentation shrinkage after drying, necessary for microhardness measurement,[8] then introducing an error in the measures.[15] In the present study, microhardness data were obtained from non-dehydrated specimens, immediately after the indentations had been performed, to avoid time-dependent influences owing to shrinkage and elastic dentin deformation.[27] The conditions of all the specimens were standardized, and since a single indentation cannot precisely reflect the dentin microhardness of a tooth as a whole,[23] measurements in triplicate were carried out at each depth of the caries lesion in order to involve all the lesion extent.

In an attempt to reproduce the dynamic process of the caries lesion development, alternating demineralisation and remineralisation,[10,11] the protocol used in the present study to induce caries-like lesions was based on a pH-cycling model,[24] adding two pH-cycling systems (12 and 16 cycles). As the proposed cariogenic challenge might be considered aggressive,[24] the immersion time in the demineralising solution was decreased, increasing the time in the remineralising solution and the exposed area of dentin.

A gradual increase in microhardness towards the deepest regions of the lesion has been observed in all the groups, which can be better visualized in Figures 1 and 2. The mineral loss that occurs along the cariogenic challenge period begins at the outer surfaces towards the inner regions, leading to a gradual decrease in microhardness, corroborating with the results described by Marquezan et al.[20] for primary dentin. For both human and bovine experimental groups, microhardness at 30 µm was lower than that of the control group, which could be explained by differences in the formation of natural and artificial caries lesions. A longer period in the natural process allows mineral deposition that can subsist for months or even years, differently from what happens in artificial caries formation, which occurs in just a few hours.

An aspect that should be considered is the influence of the organic content of natural saliva, absent in the remineralising solution used in this in vitro protocol. Moreover, laboratory models cannot produce the biological responses that may occur in vivo, such as dead tracts, mineralized tubules or secondary dentin.[8,21] However, despite these differences, the caries-like lesions process allowed an induction of a demineralised dentin with similar characteristics to those found naturally.

A comparative study of different methods for induction of artificial caries in primary dentin stated that the selection of these methods depends on the aim of the study. The pH cycles seem to

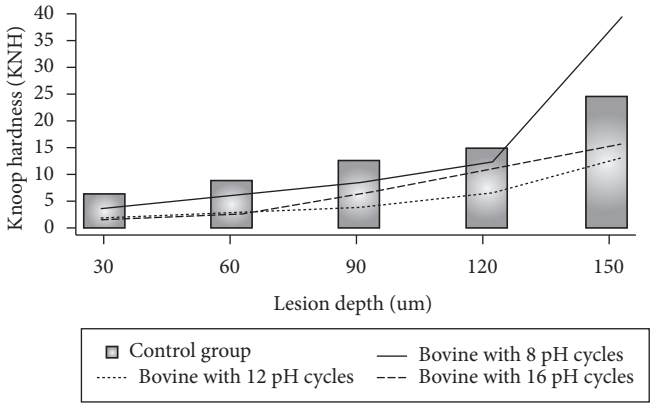


**Figure 1.** Representation of Knoop microhardness values, according to the depth and number of cycles for bovine teeth.

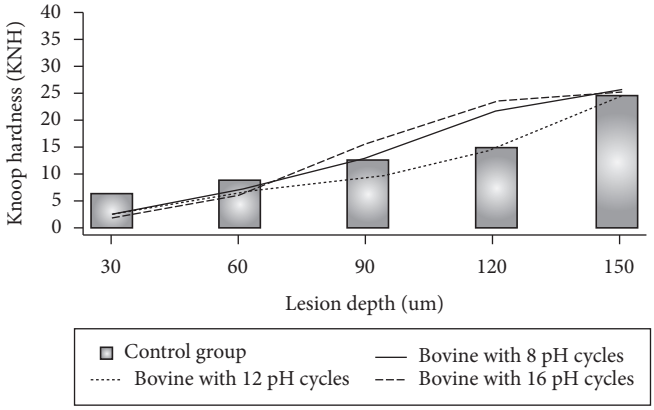


**Figure 2.** Representation of Knoop microhardness values, according to the depth and number of cycles for human teeth.

be an appropriated method to induce a lesion with characteristics that resembles affected caries dentine layer, simulating the substrate after caries removal.[20] In the present study, which used bovine and permanent teeth, similar results to Marquezan et al.[20] were found, considering the data obtained by microhardness analysis and lesion characteristics.

Amongst the bovine experimental groups, microhardness values of group B8 were the closest to those of the control group. At 150 µm, microhardness was higher than that of the control group, probably due to the gradual diffusion of acids. This 8-cycle diffusion was not sufficient to reach the subsurface at this depth, unlike the natural caries process, in which this diffusion might have occurred during a longer period. Regarding human dentin, the group H16 was the most similar to the control group, with no statistical difference at 60, 90, 120 and 150 µm. The lower number of cycles necessary to the development of caries-like lesions in bovine dentin might be partially explained by the morphology of bovine and human substrates. Bovine dentin tubules possess their larger base at the outer end,[28] which is different from those of human dentin, whose larger base is at the pulpal end.[29] This factor could have influenced the permeability of both substrates to the solutions employed in the cariogenic challenge.

Considering that groups B8 and H16 presented caries-like lesions closer to the natural ones, in relation to microhardness, it seems reasonable to state that it is possible to obtain artificial lesions similar to the natural process, using both bovine and human dentin. However, the human dentin needed a higher number of pH cycles to present lesions with similar characteristics to the natural dental caries. Thus, since the bovine dentin allows a faster de-remineralisation protocol and presents some advantageous characteristics over the human substrate, it can be considered a viable, practical and economical alternative to human dentin in dental caries research.

## CONCLUSION

As related to microhardness and within the conditions of this in vitro study, it was possible to obtain caries-like lesions both in human and bovine dentin, similar to natural caries lesions in human dentin. Future studies using this methodology may be carried out focusing on the morphology of the artificial dentin lesions. The faster de-remineralisation protocol provided by the bovine substrate suggests that it can be used as an alternative to human dentin in dental caries research.

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