Influence of sterilization method on the bond strength of caries-affected dentin

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Abstract: This study evaluated the effect of sterilization method on the bond strength of caries-affected dentin before artificial caries development and after restoration. Twelve bovine incisors were sectioned perpendicularly to their long axes at 7 mm from the amelodentinal junction. They were painted with acid-resistant nail varnish, except on an exposed coronal dentin area. Four groups were formed (n = 3) in accordance with the sterilization method used, before artificial caries development and after complete restoration: NE – no sterilization (control group); G – gamma-rays before and after; A – steam autoclave before and after; AG – steam autoclave before and gamma-rays after. For artificial caries development, dentin sections were immersed in BHI broth with *S. mutans*. After the soft carious tissue was removed, dentin was restored with Scotchbond Multi-Purpose and Filtek Z250. Next, the samples were sterilized in accordance with the methods described above and microtensile testing was performed. The data were analyzed by the Mann-Whitney test ($p < 0.05$). The G (22.7 MPa) and AG groups (16.3 MPa) were not statistically different from the NE group (17.5 MPa). Nevertheless, there were statistical differences between groups A (6.3 MPa) and NE, A and G, A and AG, G and AG. The bond strength of caries-affected dentin was not influenced by gamma-ray sterilization irrespective of whether the sterilization was performed before or after restoration.

Descriptors: Gamma rays; *Streptococcus mutans;* Dentin-bonding agents.

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Introduction

There has been increasing interest in the bonding to caries-affected dentin with adhesive systems[1] and some studies have used an *in vitro* biological caries model to produce this type of dentin to test the performance of bonding agents, as well as to develop artificial secondary caries to analyze the caries inhibition promoted by antibacterial bonding adhesives.[2-4] A biological caries model develops artificial caries using microorganisms as acid producers in a culture medium. However, the teeth must be sterilized before they are immersed in the broth culture to avoid contamination and to guarantee the specific action of the inoculated cariogenic microorganism. Thus, appropriate sterilization is required, but it should not affect the biomechanical properties of dental tissue and dental material.

The two methods most used for sterilization of extracted teeth are steam autoclave and gamma-rays.[5-7] Steam autoclave is an easy and fast sterilization method used in the dental field, but it affects enamel microhardness[8] and alters dentin structure.[7] Gamma irradiation from a cobalt-60 source, the typical method used for sterilizing hospital supplies and food, is lethal to all forms of microbial life and it sterilizes without high temperature and pressure, chemicals or gases.[9] On the enamel surface, gamma irradiation does not cause morphological changes.[9,10] The superficial hardness or enamel resistance to demineralization are not changed either,[6] but gamma irradiation can alter enamel color.[6,9] However, there are few studies that have analyzed the interaction between gamma-rays and dentin, mainly as regards bond strength.[11]

Some investigations of caries inhibition promoted by antibacterial adhesive systems[2,4] sterilized the teeth after restoration to produce artificial secondary caries. The sterilization may have affected the tooth/restoration bonding and influenced the results, but the authors did not evaluate this aspect. Furthermore, there is no research that evaluates the effect of sterilization on the dentin bond strength after restoration.

Therefore, the null hypothesis is that the sterilization method (gamma-rays and steam autoclave) does not affect the bond strength of caries-affected dentin when used before artificial caries development, to produce artificial caries-affected dentin, and after complete restoration in order to simulate secondary caries formation.

Material and Methods

Preparation of dentine samples

Twelve bovine incisors were selected for this study. After being cleaned and pumiced, they were stored in distilled water at 4°C for 30 days. The roots were sectioned at the cementoenamel junction and discarded. The crowns were sectioned perpendicularly to their long axes at 7 mm from amelodentinal junction using a low-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling (Figure 1A, B). The pulp chamber was filled with acrylic resin (Vipi Flash, Vipi Ind. Com., Pirassununga, SP, Brazil). The prepared teeth were painted with acid-resistant nail varnish (Colorama, CEIL Coml. Exp. Ind. Ltda., São Paulo, SP, Brazil), except for a coronal dentin surface area of 14.4 mm², left for exposure to caries development, (Figure 1C). Four groups were distributed (n = 3) in accordance with the sterilization method before artificial caries development and after complete restoration: NE – no sterilization (control group); G – gamma-rays before and after; A – steam autoclave before and after; AG – steam autoclave before and gamma-rays after.

Sterilization methods

The dentin samples were fixed on the lids of glass container vessels with orthodontic wire (Figure 1D) and kept immersed in 50 ml of sterile distilled water until they were sterilized. Gamma irradiation was performed in a gamma radiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottawa, Canada) for 18 hours and 58 minutes at 27°C with a 14.5 kGy dose[12] in the same glass container (Figure 1E). The irradiation time was determined taking into consideration the correction for radioactive decay of the gamma-ray source. Steam autoclaving (Phoenix Ind. Brasileira, Araraquara, SP, Brazil) was performed for 15 min at 121°C.[10]

Artificial caries development

The samples previously sterilized in accordance with their respective group were transferred to another glass container containing 50 ml of sterile brain-heart infusion broth (Becton Dickinson and Company, Sparks, MD 21152, USA), supplemented with yeast extract (Himedia Laboratories PVT Ltd., Mumbai, India), 0.5% glucose (Synth, LabSynth, Diadema, SP, Brazil), 1% sucrose (Synth, LabSynth, Diadema, SP, Brazil) and 2% *S. mutans* (UA 159)[13] (Figure 1F, G). The concentration of this bacterial suspension was determined by measuring absorption at 660 nm (A_{660}).[14] To adjust the number of viable bacteria to A_{660}, the number of colony-forming units (CFUs) per milliliter of bacterial suspension was determined with the use of standard spreading techniques at carious optical densities.[14] Inoculation occurred only on the first day of the experiment, but the culture media was renewed every 48 h during 14 days.[13] Contamination in the media was verified every 48 h by means of Gram staining.

Carious dentine removal and restoration

The soft and infected carious tissue was removed with #8 round tungsten carbide burs (KGSorensen, Barueri, SP, Brazil) in a slow-speed handpiece (Figure 1H). The tactile sensation criterion with dental explorers and visual examination were used to identify the caries-affected dentin.[1] Immediately after, Adper Scotchbond Multi-Purpose (3M-ESPE, St. Paul, MN, USA) was applied in accordance with the manufacturer's instructions (Table 1) and the bonded surfaces were coupled with a hybrid resin composite (Filtek Z250, 3M-ESPE, St. Paul, MN, USA lot1370A1) that was applied in 2-mm-thick increments and polymerized in a quartz-tungsten-halogen light-curing unit at 700 mW/cm² (Tri-Light, 3M-ESPE, St. Paul, MN, USA) to form 4-mm-thick cores. Each increment was light-cured for 20 s. The teeth with composite build-ups were stored in

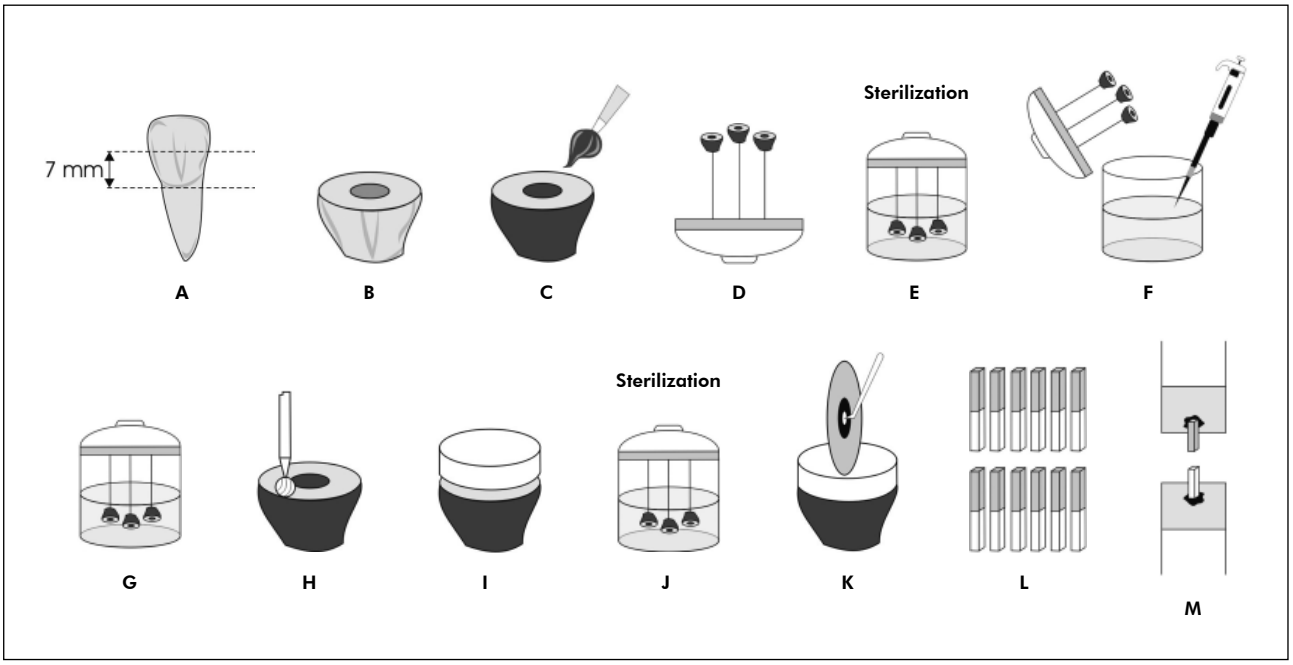

Figure 1 - (A) Section perpendicular to the long axis of the tooth at 7 mm from the amelodentinal junction. **(B)** Flattened exposed dentin surface. **(C)** Samples painted with acid-resistant nail varnish, leaving 14.4 mm² of dentin surface area to be exposed to artificial caries development. **(D)** Samples fixed with orthodontic wires on the lids of glass container vessels. **(E)** Sterilization. **(F)** Inoculation of medium with *Streptococcus mutans* cultures. **(G)** Artificial caries development. The samples were transferred into fresh media every 48 h. **(H)** Soft and infected carious tissue removal with round tungsten carbide burs. **(I)** Restoration. **(J)** Sterilization. **(K)** Cross-section performed perpendicularly to the restoration. **(L)** Beams obtained after the cutting procedures. **(M)** Specimen broken after microtensile testing.

distilled water at 37°C for 24 h (Figure 1I). After complete restoration, the specimens were sterilized again, in accordance with the method of each group, in the same manner reported previously (Figure 1J).

Microtensile bond testing

Each specimen was cross-sectioned perpendicularly to the resin-dentin interface with a slow-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling, yielding square beams of approximately 1 mm², in accordance with the non-trimming version of the microtensile bond test reported by Shono *et al.*[15] (1999) (Figure 1K). Beams at specimen peripheries were discarded, yielding 10-12 bonded beams per tooth (Figure 1L).

Before bond-testing, the exact beam dimensions were measured to the nearest 0.01 mm with a digital caliper (Starret 727-6/150; Starret, Itu, SP, Brazil) to calculate bond strength, which was expressed in MPa. The beams were stressed to failure under tension in a Bencor Multi-T device (Danville Engineering, San Ramon, CA, USA) in a universal testing machine (Instron model 4411; Canton, MA, USA) at a crosshead speed of 0.5 mm/min (Figure 1M).

The failure mode of each specimen was determined under a stereomicroscope (Meiji EMZ-TR, Meiji Techno Co. Ltd., Tokyo, Japan) at x60 magnification and designated as an adhesive, mixed or cohesive failure in either adhesive or resin composite.

Statistical analysis

The data were analyzed by the Mann-Whitney Test to examine the effect of sterilization method on bonding strength. Statistical significance was set at $\alpha = 0.05$.

Results

Statistical analysis of the bond strength data derived for both methods of sterilization before artificial caries development and after complete restoration showed that the microtensile bond strengths of caries-affected dentin/resin were affected by the method used for sterilization ($p < 0.05$) (Table 2).

Specimens sterilized with gamma-rays before artificial caries development and after complete restoration (G) exhibited significantly higher bond strengths than specimens sterilized with steam autoclave before and after (A). Group G showed higher bond strength values than group AG, but neither of them were statistically different from the control group (no sterilization – NE), with $p = 0.06$ and $p = 0.79$, respectively (Table 2).

The failure mode obtained in group A was 75% mixed, 8.4% adhesive, and 16.6% cohesive in the

Table 1 - Composition and mode of application of the total etch adhesive system used in this study (Adper Scotchbond Multi-Purpose – 3M-ESPE).

Composition	Mode of application
Acid Conditioner: 37% Phosphoric acid (Batch:7523 5FB)	Apply Acid Conditioner on dentin for 15 s, rinse for 15 s, dry gently for 2 s. Leave moist.
Primer: water, ethanol, HEMA[a], copolymer of polyalkanoic acid (Batch: 3008)	Apply *Primer* to dentin. Dry gently for 5 s.
Adhesive: BisGMA[b], HEMA, dimethacrylate, initiators (Batch: 7583)	Apply *Adhesive* to dentin. Light cure for 10 s.

a: hydroxyethyl methacrylate. b: bisphenol A diglycidyl methacrylate.

Table 2 - Microtensile bond strengths of the Adper Scotchbond Multi-Purpose adhesive system to caries-affected dentin in which sterilization methods were applied before artificial caries development and after complete restoration.

Treatment	Microtensile Bond Strength*
No-sterilization *(NE)*	17.5 ± 10.8 (36)[a, c]
Gamma-rays *(G)*	22.7 ± 11.3 (34)[a]
Steam autoclave *(A)*	6.3 ± 4.4 (12)[b]
Steam autoclave + Gamma-rays *(AG)*	16.3 ± 9.0 (33)[c]

* Values are presented as mean ± standard deviation in MPa. Groups with the same superscripts are not statistically different ($p > 0.05$). The numbers of specimen beams tested are enclosed in parentheses.

adhesive. In group NE, the failure mode was 68% mixed, 4% adhesive, and 24% cohesive in the adhesive; and 4% cohesive in the resin. In group G, it was 70.6% mixed, and 29.4% cohesive in the adhesive. In group AG, it was 57.6% mixed, 39.4% cohesive in the adhesive, and 3% adhesive.

Discussion

The null hypothesis was not accepted since the sterilization method affected the bond strength of the caries-affected dentin, when used before artificial caries development to produce artificial caries-affected dentin, and after complete restoration, in order to simulate secondary caries formation.

The dose of gamma irradiation recommended for medical industry products is 25 kGy.[16] However, the present study used a 14.5 kGy dose and it was able to sterilize without jeopardizing bond strength. Likewise, Rodrigues *et al.*[12] (2005) demonstrated that a 14.5 kGy dose sterilized dental enamel blocks and no microbial growth was observed. The physical and mechanical properties of the material are dose-dependent,[17] thus, the lower the dose used for material sterilization (with no microbial growth), the less the alteration of their properties.

Specimens sterilized with gamma-rays before artificial caries development and after complete restoration exhibited significantly higher bond strengths than the specimens sterilized with steam autoclave before and after. Although there are no investigations about the effect of sterilization method on bond strength of dentin after restoration, the lower bond strength values in group A can be due to alteration in the dentin structure caused by steam autoclaving. FTIR spectra have demonstrated a change in the mineral component of dentin discs after autoclave sterilization.[7] Furthermore, an elevated temperature and high level of moisture were detrimental to adhesion, favoring water sorption, leaching resin components, disrupting polymer interchain hydrogen bonds, causing a plasticization effect.[18,19] These factors probably explain why several beams were debonded at the resin/dentine interface during the cross-sectioning of Group A beams.

This study corroborates the investigation by Sperandio *et al.*[11] (2001), who observed that there was no statistical difference between non-sterilized and gamma-ray sterilized groups. Although the dentin surface morphology was not affected by gamma-rays[11] nor were any dentin structural changes detected,[7] some investigations demonstrated that sterilization with gamma-irradiation may cause molecular fragmentation via chain scission of the collagen peptide chains.[17,20] Peptide fragmentation can lead to denaturation (nativity loss) of the collagen molecule that may adversely affect the mechanical properties.[16,17] However, the presence of glucose in glucose-incorporated collagen films causes a "strengthening" effect due to apparent crosslink formation by the induction of free radical sites within sugar molecules.[21] The crosslink formation may preserve the native state of the collagen molecule, maintaining greater strength and durability compared with uncrosslinked collagen.[16]

Enamel composition consists of 96% mineral (hydroxyapatite crystals) and 4% organic material and water.[22] Because there are no collagen fibrils, no morphological changes were found in the enamel surface, nor were there any changes in its hardness or its resistance to demineralization.[6,10] The authors speculate that the presence of glucose and sucrose in the broth for developing the artificial caries might have influenced the higher bond strength values in the G and AG groups compared with the A group, due to the formation of glucose-derived crosslinks. Furthermore, this effect of collagen "strengthening" by gamma-rays may also be the cause of the lack of statistical difference between the G and AG groups compared to the control group. Additionally, it could be responsible for the difference found between the G and AG groups, since only group G was sterilized twice with gamma-rays.

Obviously, restorations will not be sterilized clinically, but this study was developed in order to select a sterilization form that could be used in an *in vitro* biologic challenge caries test, because studies have been developed without testing the effect of sterilization. However, further investigations are necessary to confirm the glucose effect on teeth sterilized by gamma-rays and their influence on dentin bond strength using an *in vitro* biologic model as cariogenic challenge. Similarly, it is necessary to in-

vestigate the effect of gamma-rays on dental material properties.

Conclusion

Based on the results of this study, it could be concluded that the gamma rays method used before caries development and after restoration had no influence on the bond strength of caries-affected dentin, but steam autoclave did present a negative influence.

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