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Laboratory Research

Transdentinal Cytotoxicity of Carbodiimide (EDC) and Glutaraldehyde on Odontoblast-like Cells

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Clinical Relevance

Carbodiimide (EDC) and glutaraldehyde are cross-linking agents capable of increasing resin-dentin bond durability. This study showed that these substances might be safely applied on acid-etched dentin since they have no transdentinal cytotoxic effects on cultured odontoblast-like cells.

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SUMMARY

Objective: To evaluate the transdentinal cytotoxicity of three different concentrations of carbodiimide (EDC) or 5% glutaraldehyde (GA) on MDPC-23 cells.

Methods: Seventy 0.4-mm-thick dentin disks obtained from human molars were adapted to artificial pulp chambers. MDPC-23 cells were seeded on the pulpal surface of the disks. After 48 hours, the occlusal dentin was acid-etched and treated for 60 seconds with one of the

following solutions (n=10): no treatment (negative control); 0.1 M, 0.3 M, or 0.5 M EDC; 5% GA; Sorensen buffer; or 29% hydrogen peroxide (positive control). Cell viability and morphology were assessed by methyltetrazolium assay and scanning electron microscopy (SEM), respectively. The eluates were collected after the treatments and applied on MDPC-23 seeded in a 24-well plate to analyze cell death, total protein (TP), and collagen production. The last two tests were performed 24 hours and seven days after the challenge. Data were analyzed by Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

Results: EDC at all test concentrations did not reduce cell viability, while 5% GA did increase cell metabolism. Cell death by necrosis was not elicited by EDC or 5% GA. At the 24-hour period, 0.3 M and 0.5 M EDC reduced TP production by 18% and 36.8%, respectively. At seven days, increased TP production was observed in all groups. Collagen production at the 24-hour period was reduced when 0.5 M EDC was used. After seven days, no difference was observed among the groups. SEM showed no alteration in cell morphology or number, except in the hydrogen peroxide group.

Conclusions: Treatment of acid-etched dentin with EDC or GA did not cause transdentinal cytotoxic effects on odontoblast-like cells.

INTRODUCTION

The biodegradation of resin-dentin bonds is a complex process that involves the leaching of unpolymerized monomers that infiltrated the demineralized dentin matrix and the enzymatic cleavage of exposed collagen fibrils.[1,2] Therefore, the resistance of the adhesive interface components against degradation determines its stability and durability.[1,3-5]

Collagen fibrils that are not encased by monomers during the bonding procedure,[6,7] as well as those exposed by the polymer degradation over time, are highly susceptible to enzymatic hydrolysis.[8-12] Matrix metalloproteinases (MMPs) and cysteine cathepsins have been identified in sound and caries affected-dentin[8,12-15] and are allegedly responsible for the disappearance of portions of the hybrid layers.[11,16,17] Thus, improving collagen's resistance against enzymatic degradation and inactivating these proteases are important steps to enhance the quality and longevity of dentin bonding.

The treatment of demineralized dentin with EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) or glutaraldehyde (GA) increases the mechanical properties of dentin collagen[18] and inhibits MMPs[19,20] by creating new cross-links among protein peptide chains. EDC is capable of forming covalent peptide bonds between peptide chains by activating the free carboxyl group of glutamic and aspartic acids,[21,22] while GA reacts with the ε-amino groups of lysyl or hydroxylysyl residues to induce the formation of intra- and intermolecular cross-links.[23,24]

Although the effects of EDC and GA have been demonstrated on collagen biomodification,[18,25] rendering collagen more resistant to degradation by improving its mechanical properties, such as elastic modulus and ultimate tensile strength, and MMP inhibiton,[19,20] there is little information available about their cytotoxicity. The carbodiimides have shown better results than has GA in terms of the biocompatibility on U937 macrophage-like cells,[26] rat ocular cells/tissue,[27] and corneal endothelial cells.[28] However, there are no studies that have tested the transdentinal cytotoxicity of these cross-linking agents on odontoblast-like cells beneath dentin to determine and confirm if these substances are safe when applied on acid-etched dentin.

Thus, the aim of this study was to evaluate the transdentinal cytotoxicity of different concentrations of EDC or 5% GA on odontoblast-like cells. The tested null hypothesis was that the treatment of acid-etched dentin with cross-linkers for 60 seconds does not exert cytotoxic effects on the target cells.

METHODS AND MATERIALS

Preparation of Dentin Disks and Permeability Reading

Seventy sound human third molars were obtained after approval was received by the Ethics Committee of the School of Dentistry at Araraquara–UNESP; these teeth were stored in 0.12% thymol solution at 4°C. The teeth were used within three months after extraction. One 0.5-mm-thick dentin disk was obtained from the mid-coronal dentin of each tooth using a precision cutting machine equipped with a water-cooled diamond saw (Isomet 1000, Buehler Ltd, Lake Bluff, IL, USA). The disks were carefully examined with a stereoscopic microscope (SZX7, Olympus, São Paulo, SP, Brazil) to confirm the absence of enamel islets and defects resulting from pulp horn projections. Then the occlusal side of the disks was manually finished with wet 320-grit

silicon carbide paper to reach a final thickness of 0.4 mm, as measured with a digital caliper accurate to the nearest 0.01 mm (Mitutoyo South Americana Ltd, Suzano, SP, Brazil).

Dentin permeability was determined to permit a homogeneous distribution of the dentin disks into the groups. The smear layer produced on both sides of the disks was removed by application of 0.5 M ethylenediamine tetraacetic acid (pH 7.4) for 60 seconds, followed by abundant rinsing with deionized water. For determination of the hydraulic conductance, the disks were individually placed in *in vitro* pulp chambers (IVPCs) modified from Hanks and others.[29] A metallic cannula connected the IVPC to a 180-cm column of water. The disk remained under this pressure for five minutes, after which time the movement of a microbubble introduced through the cannula was recorded over the course of one minute, and the obtained values were transformed into conductance values. Then the dentin disks were allocated into seven groups (n=10) in such a way that the dentin permeability was statistically similar among the groups (analysis of variance, $p>0.05$).

MDPC-23 Cell Seeding on Dentin Disks

MDPC-23 cells were cultivated in Dulbecco Modified Eagle Medium (DMEM; Sigma Aldrich Corp, St Louis, MO, USA) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine (Gibco, Grand Island, NY, USA) in a humidified incubator with 5% CO_2 and 95% air at 37°C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The cells were subcultured each three days until the number of cells necessary to perform the study was reached.

To simulate clinical conditions, the smear layer was recreated on the occlusal side of each disk with a 600-grit silicon carbide paper for 10 seconds. Then the disks were placed in modified IVPCs and were both sterilized in ethylene oxide. MDPC-23 cells (3×10^4) were seeded on the pulpal side of the dentin disks (0.28 cm^2). In order to accomplish that, the IVPCs were placed in an inverted position (pulp side up) into the compartments of 24-well plates (CO-STAR 3595, Corning Incorporated, Corning, NY, USA) and maintained in an incubator with 5% CO_2 and 95% air at 37°C for 48 hours to ensure adherence of cells. After this time, the IVPCs were carefully removed from the compartment and returned to the same compartment with the occlusal side up to receive the treatment solutions.

Application of the Cross-linking Solutions

Four treatment solutions (10 disks per solution) were prepared by diluting the cross-linking agent in Sorensen buffer (pH 6.2): 0.1 M EDC (pH 6.04); 0.3 M EDC (pH 5.98); 0.5 M EDC (pH 5.93), and 5% GA (pH 5.8). In addition, three more conditions were tested (n=10): Sorensen buffer only, 29% hydrogen peroxide (positive control), and no treatment (negative control). The EDC and GA concentrations were chosen based on satisfactory results observed in previous studies regarding MMP inhibition and increase in collagen mechanical properties.[19,20,30,31]

The occlusal surface of the dentin disks was etched with 35% phosphoric acid (Scotchbond etchant, 3M ESPE, St Paul, MN, USA) for 15 seconds, carefully rinsed with deionized water for 10 seconds, and blot-dried with sterilized cotton pellets. Then 20 μL of the predetermined treatment solution (cross-linking solutions, Sorensen buffer, or controls) was applied for 60 seconds[19,20,31]; this step was followed by water rinsing and blot-drying. All procedures were performed in a vertical laminar flow chamber to prevent contamination, and immediately afterward, the IVPCs were placed again in a CO_2 incubator for an additional 24 hours.

Analysis of Cell Viability (Methyltetrazolium [MTT] Assay)

Eight out of the 10 disks of each group were randomly selected for cell viability analysis using the MTT assay. The eluates (DMEM + products that diffused through the dentin disks) from each well were collected and frozen for subsequent analysis of type of cell death as well as type 1 collagen and total protein production. After 24-hour incubation, the disks were removed from the IVPCs and individually placed in the sterilized wells of new 24-well plates with the pulpal side containing the MDPC-23 cells turned upward. Then 900 μL of fresh DMEM and 100 μL of MTT solution (Sigma-Aldrich) (5 mg/mL in sterile phosphate-buffered saline [PBS]) were placed in contact with the disks. The cells were incubated with the MTT solution at 37°C for four hours. Next, the MTT solution was aspirated and 400 μL of acidified isopropanol solution was added (0.04 N HCl) in each well to dissolve the violet formazan crystals, producing a homogeneous purple solution.

Three 100-μL aliquots of each well were transferred to wells of 96-well plates (Costar Corp, Cambridge, MA, USA) and read at 570-nm wavelengths with an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Plate, Nanshan

District, Shenzhen, Gandong, China). The values obtained from the three aliquots were averaged to provide a single value for each sample.

Scanning Electron Microscopy (SEM) Cellular Morphology Analysis

Two disks from each group were randomly selected and prepared for SEM analysis. The cells were fixed in 2.5% GA (Sigma-Aldrich) in PBS for one hour at room temperature. Next, the glutaraldehyde was aspirated and the cells were rinsed with PBS, postfixed with 1% osmium tetroxide (Electron Microscopy Science, Fort Washington, PA, USA) for one hour, and rinsed again with PBS, followed by dehydration with ascending series of water–ethanol solutions (30%, 50%, 70%, 95%, and 100%) two times for 60 minutes each.

The cells were immersed for 60 minutes (three 20-minute changes) in 1,1,1,3,3,3-hexamethyldisilazane (ACROS Organics, Morris Plains, NY, USA). Finally, the specimens were mounted on metallic stubs and stored in a desiccator for 24 hours and sputter-coated with a gold layer (SDC 050; Bal-Tec AG, Balzers, Germany), and their morphology was examined with SEM (DSM 960, Carl Zeiss Inc, Oberköchen, Germany).

Cell Membrane Damage Measurements (Cell Death)

To analyze the cell membrane damage the Live/Dead Cell Viability/Cytotoxicity Kit (Invitrogen, San Francisco, CA, USA) was used. This assay uses the fluorescence probe ethidium homodimer-1 (EthD-1) that binds to DNA bands only in cells with cell membrane rupture. The second probe was the Calcein AM (CA), which is hydrolyzed by cytoplasmic esterases in viable cells. MDPC-23 cells were seeded in a 24-well plate (n=5) and exposed to the eluate collected after the dentin treatment for 24 hours. Then the supernatant was centrifuged (4000 rpm for two minutes), resuspended with DMEM, and returned to its original well. The plate was centrifuged (4000 rpm for two minutes) in order to allow the cells to precipitate, and the cells were incubated with 2 mM CA and 4 mM Eth-1 and Hoechst (1:5000) for nuclear staining, over the course of 15 minutes. The fluorescence was analyzed by an In Cell Analyzer 2000 (GE Healthcare Life Sciences, Freiburg, Germany) in six fields per well. The percentage of dead (positive Eth-1 staining) and live (positive CA staining) cells were calculated from the cells stained with Hoechst with the software In Cell Investigation (GE Healthcare Life Sciences), and the average value per well was used for statistical analysis.

Total Protein (TP) Production

Total protein (TP) production was evaluated according to the Read and Northcote protocol (1981), as previously described by Basso and others.[32] The eluate collected after dentin treatment (n=5) was placed in contact with MDPC-23 cells seeded in a 24-well plate for 24 hours. TP production was also evaluated seven days after placement in contact with EDC solutions. In both instances, the eluates were frozen for total collagen production detection and the cells were washed three times with 1 mL PBS at 37°C, and 1 mL of 0.1% sodium lauryl sulfate (Sigma-Aldrich Corp) was added to each well for 40 minutes at room temperature to produce cell lysis. After homogenization, an aliquot of 1 mL of each well was transferred to Falcon tubes, while the blank tube received 1 mL of distilled water (TP protocol was performed in 24-well plates, not in Falcon tubes). Next, the Lowry reagent solution (Sigma-Aldrich Corp) was added (1 mL) to all samples, the tubes were agitated for 10 seconds, and after 20 minutes, 500 mL of Folin-Ciocalteau's phenol reagent solution (Sigma-Aldrich Corp) was added to each sample and homogenized. Three 100-μL aliquots of each tube were transferred to a 96-well plate after 30 minutes, and the absorbance was read at 655 nm in an ELISA plate reader (Thermo Plate). The average of the three values was used for statistical analysis. Absorbance values were transformed into percentage, and the average of the control group was considered 100% of TP production.

Collagen Production

Collagen production by MDPC-23 cells was evaluated 24 hours and seven days after contact with EDC or GA solutions. The 24-hour and seven-day eluates were thawed and a 200-μL aliquot from each tube was transferred to a sterilized well of new 24-well plates. Then 500 μL of 0.1% Sirius Red dye (Sirius Red powder in picric acid, Sigma-Aldrich) was added to each well and the plate was incubated with shaking for one hour at 37°C. The content of each well was placed in 1.5-mL tubes, thus enabling sample centrifugation at 12,000 rpm. Supernatant was discarded and the precipitate was rinsed with 750 μL of 0.01 M HCl to each tube. Once again, samples were centrifuged for 10 minutes, the supernatant was discarded, and 250 μL of 0.5 M NaOH was added to solubilize the precipitate. After

that, three 100-μL aliquots were transferred to a 96-well plate and read at 555 nm in a 96-well plate reader (Synergy H1 Hybrid Reader, Biotek, Winooski, VT, USA). The values obtained from the three aliquots were averaged to provide a single value for each sample. Total production of collagen was calculated based on a standard curve performed with predetermined concentrations of this protein.

Statistical Analysis

Data from the response variables (cell viability, cell death by necrosis, production of TP, and type I collagen) were not normally distributed and were analyzed by the application of Kruskal-Wallis and Mann-Whitney nonparametric tests. The latter was used for pairwise comparison between groups. For all statistical tests, $p < 0.05$ was taken to be statistically significant.

RESULTS

MDPC-23 cell viability and death by necrosis after contact with the transdentinal diffused components of different concentrations of EDC or 5% GA are shown in Figure 1. None of the EDC concentrations differed from the negative control or Sorensen buffer with regard to cell viability (Figure 1a). Only 5% GA increased the cell viability, although it was not statistically different from the EDC solutions, regardless of their concentrations. Cell death by necrosis (Figure 1b) was not increased by any of the investigated EDC concentrations or by 5% GA. The percentage of cell death varied from 0.5% to 1.9% for EDC groups. Variations in TP production are expressed as a percentage in Table 1; all of the values were calculated based on 24-hour–control. At 24 hours, only 0.3 M and 0.5 M EDC negatively interfered with TP production. Compared to the control (101.2%), the cell viability for these groups was 82.0% and 63.2%, respectively (Table 1, column). Lack of effect on TP production was seen for 0.1 M EDC and 5% GA, which did not differ from the control. However, at seven days, a significant increase in TP production occurred in all groups, compared to the 24-hour period (Table 1, rows). Collagen production at the 24-hour period was significantly reduced only by 0.5 M EDC in comparison to the negative control. Significant increase in the production of collagen from 24 hours to seven days was seen only for 0.5 M EDC (Table 2). The SEM analyses confirmed the results obtained with the MTT and Live/Dead assays. A large number of odontoblast-like MDPC-23 cells remained adhered to the dentin disks in all groups (Figure 2a-g), except in the positive control (29% hydrogen peroxide, Figure 2h). In the latter, wide cell-free zones and large areas of residual fragments of dead cells were observed. In the negative control, EDC, and GA groups, the cultured cells were near to confluence and exhibited abundant cytoplasm with numerous thin cytoplasmic projections that seemed to be adhering the cells to the dentin substrate (Figure 2b).

DISCUSSION

This study evaluated the transdentinal cytotoxicity of EDC solutions on odontoblast-like cells. The IVPCs containing dentin disks were used in order to simulate extremely deep cavities in which MDPC-23 cells were indirectly exposed to the tested solutions. This *in vitro* test simulates the *in vivo* condition in which the odontoblasts are the first cells to be in contact with components that have diffused through the dentin.[33] The sound dentin is a tubular mineralized matrix capable of protecting the pulp, even at a thickness as thin as 0.5 mm.[29,33] To increase the probability of the cytotoxic potential of each solution, 0.4-mm-thick disks were used in the present study.

EDC has been investigated in dentistry as a cross-linking agent that when applied directly to demineralized collagen increases its resistance against enzymatic degradation[18] and inhibits the endogenous MMPs of dentin.[19,20,34] Additionally, EDC is able to preserve the resin-dentin bond over time.[35] In the medical field, EDC has been used to reinforce different structures for tissue engineering applications and has shown satisfactory biocompatibility. This agent may support drug delivery system development,[36] bioprosthetic heart valve construction,[26] preparation of collagen scaffolds,[37] and a large number of other purposes.[38-41] However, these procedures use EDC to treat biomaterial structures prior to their contact with cells/tissues. The same is not observed when EDC is applied on dentin, since its fluid-filled tubular and porous structure allows EDC to diffuse across dentin to reach the odontoblast-like cells following its application. However, dentin thickness is known to dissipate the concentration of potential toxins over distance.[42]

GA is used in many different processes as a fixative, cross-linking, and disinfecting agent. As is the case for EDC, GA participates in the construction of bioprosthetic heart valves,[26,43] inhibits proteases,[31] and modifies gelatins and other materials and tissues.[26,44,45] In dental treatments, GA has been used as a desensitizer that reacts with plasma proteins in dentin fluid to precipitate them, blocking

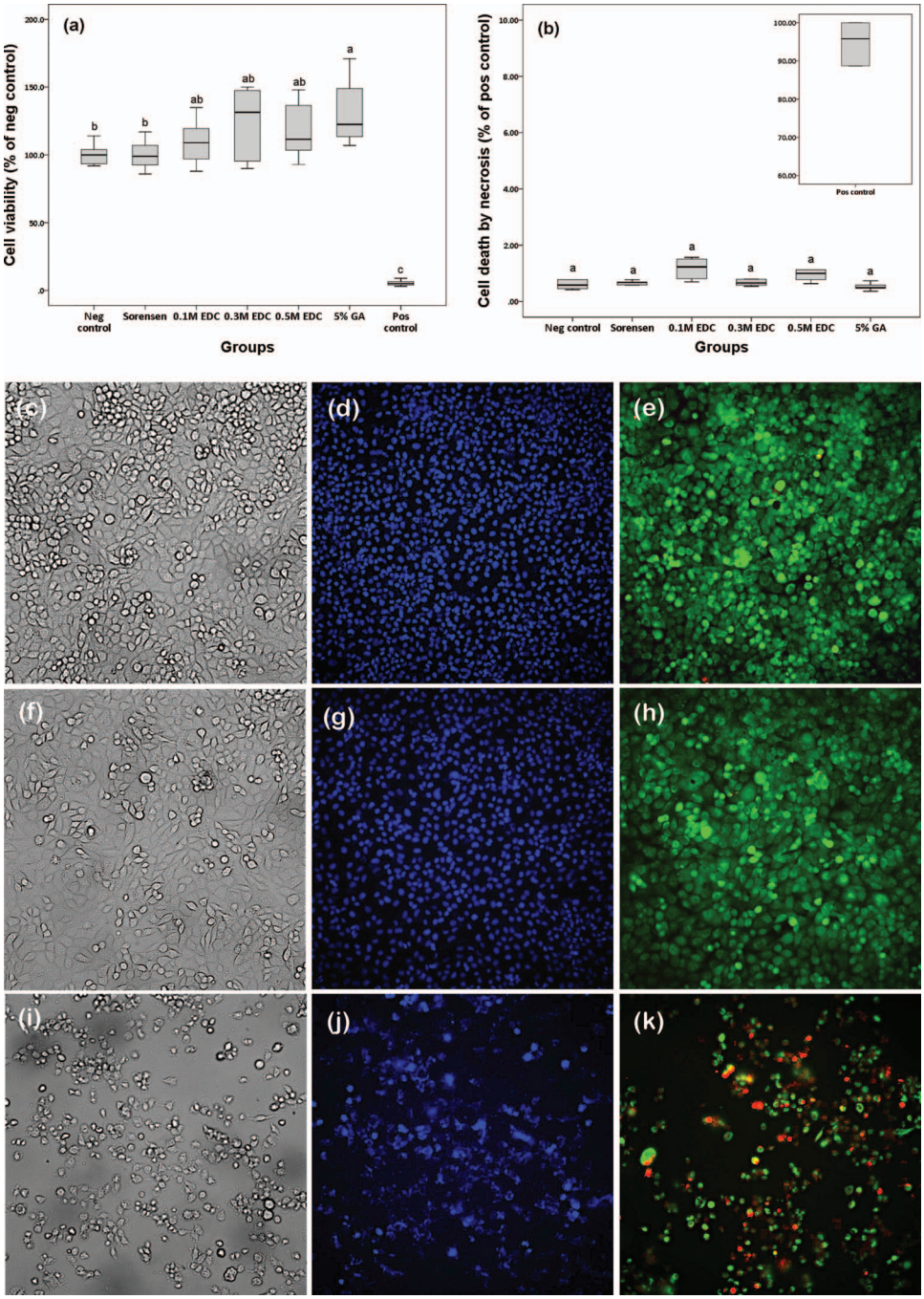

Figure 1. *Response of MDPC-23 cells after transdentinal contact with different concentrations of EDC. (a) Percentage of cell viability (MTT assay) related to the negative control (=100% of viable cells). (b) Percentage of necrotic dead cells related to the positive control (=100% of cell death). Groups identified by the same letter do not differ statistically (Mann-Whitney,* $p > 0.05$*). (c-k) Representative confocal images of MDPC-23 cells co-stained with LIVE/DEAD® assay Kit after contact with Sorensen buffer (c, d, and e), 0.5 M EDC (f, g, and h), and positive control (i, j, and k). Live cells fluoresce green on Calcein dye uptake, and necrotic cells fluoresce red on ethil homodimer-1 uptake. Hoechst dye (blue) shows cell nucleus, representing total cell number observed in the white field. Magnification 20×.*

dentin tubules.[46-48] Wiegand and others[49] showed that a GA-containing dentin desensitizer applied on 1.0-mm-thick dentin specimens under simulated pulpal flow conditions did not present toxic effects on L-929 fibroblasts. The same was observed by Camps and others[50] using 0.5-mm-thick dentin slices. In agreement with those authors, the results of this study showed that 5% GA did not exert harmful effects on MDPC-23 cells when applied on 0.4-mm-thick dentin disks.

Table 1: *Production of Total Protein (% of Negative Control at 24 hours) by MDPC-23 Cells After Transdentinal Contact with Different Concentrations of Carbodiimide (EDC)*[a]

Groups	Period of Evaluation		
	24 h		7 d
Negative control	101.2 (100.7-101.2) A	*	250.4 (241.0-254.2) BC
Sorensen	108.8 (105.8-111.9) A	*	262.4 (254.8-263.6) AB
0.1 M EDC	95.1 (81.5-97.7) AB	*	244.1 (243.5-245.4) C
0.3 M EDC	82.0 (82.0-84.0) BC	*	263.6 (261.7-267.4) AB
0.5 M EDC	63.2 (59.7-64.7) C	*	256.1 (248.5-262.4) BC
5% GA	87.0 (78.9-87.0) AB	*	271.8 (263.6-272.4) A

Abbreviation: GA, glutaraldehyde.
[a] *Numbers are median (percentile 25-percentile 75), n=5. Within each column, groups followed by the same letter are not statistically different (Mann-Whitney, p>0.05).*
** Indicates statistically significant difference between periods of evaluation (Mann-Whitney, p>0.05).*

In the present study, the cross-linking solutions were applied for one minute on the occlusal side of acid-etched dentin disks, which were then rinsed with deionized water prior to the analysis of the solutions' cytotoxic effects. The MTT assay revealed that none of the EDC concentrations differed from the negative control or Sorensen buffer with regard to cell viability. While 5% GA was able to slightly increase the cellular metabolism, the good results may have been favored by the water rinsing that removed the residual reagents after the treatment.

Cell death by necrosis occurs after irreversible cell damage generated by a chemical, physical, or biological injury. The Live/Dead assay data showed that EDC and GA presented no difference with regard to the percentage of cell necrosis compared to the negative control group (no treatment). In contrast, 29% hydrogen peroxide (positive control) induced between 90% and 100% cell death by necrosis. Corroborating cell viability and cell death findings, SEM observations, that were used in this study as a descriptive and qualitative method, showed no significant alterations on the phenotypical characteristics and/or number of cells to all groups, except for H_2O_2, the positive control.

The TP and collagen production were analyzed 24 hours and seven days after the application of the treatment solutions. Only 0.3 M and 0.5 M EDC significantly reduced 24-hour TP production compared to the control group. However, this reduction was no longer significant after seven days, which suggests that if EDC caused any alteration in the cell function it was reversible in a short period of time and did not affect cell viability. Treatment with 5% GA had been similar to the control group (no treatment) at 24 hours; it was not observed after seven days when GA, 0.3 M EDC, and Sorensen buffer treatments increased TP production. The analysis of collagen concentration in the eluate showed that 0.5 M EDC reduced collagen production 24 hours after its application. However, after seven days, no difference was observed in any treated group when compared to the control.

The results from the different tests are in agreement, showing that EDC or GA applied on acid-etched dentin for 60 seconds and then rinsed

Table 2: *Type I Collagen Production (% of Negative Control at 24 hours) by MDPC-23 Cells Exposed to Cell Culture Medium Beneath Acid-etched Dentin Treated with Carbodiimide (EDC) or Glutaraldehyde (GA)*[a]

Groups	Period of Evaluation		
	24 h		7 d
Negative control	100.4 (97.1-103.0) A	n.s.	100.3 (96.3-103.2) AB
Sorensen	102.0 (97.1-104.8) A	n.s.	94.9 (91.0-97.4) B
0.1 M EDC	97.7 (91.1-104.7) AB	n.s.	89.7 (80.0-97.5) B
0.3 M EDC	106.7 (103.1-110.7) A	n.s.	92.9 (92.4-93.3) B
0.5 M EDC	83.8 (74.2-91.4) B	*	98.1 (96.3-100.8) B
5% GA	94.2 (87.2-102.6) AB	n.s.	105.3 (104.2-106.5) A

[a] *Numbers are median (percentile 25-percentile 75), n=5. Within each column, groups followed by the same letter are not statistically different (Mann-Whitney, p>0.05).*
** Indicates statistically significant difference between periods of evaluation, while n.s. (not significant) indicates absence of significance (Mann-Whitney, p>0.05).*

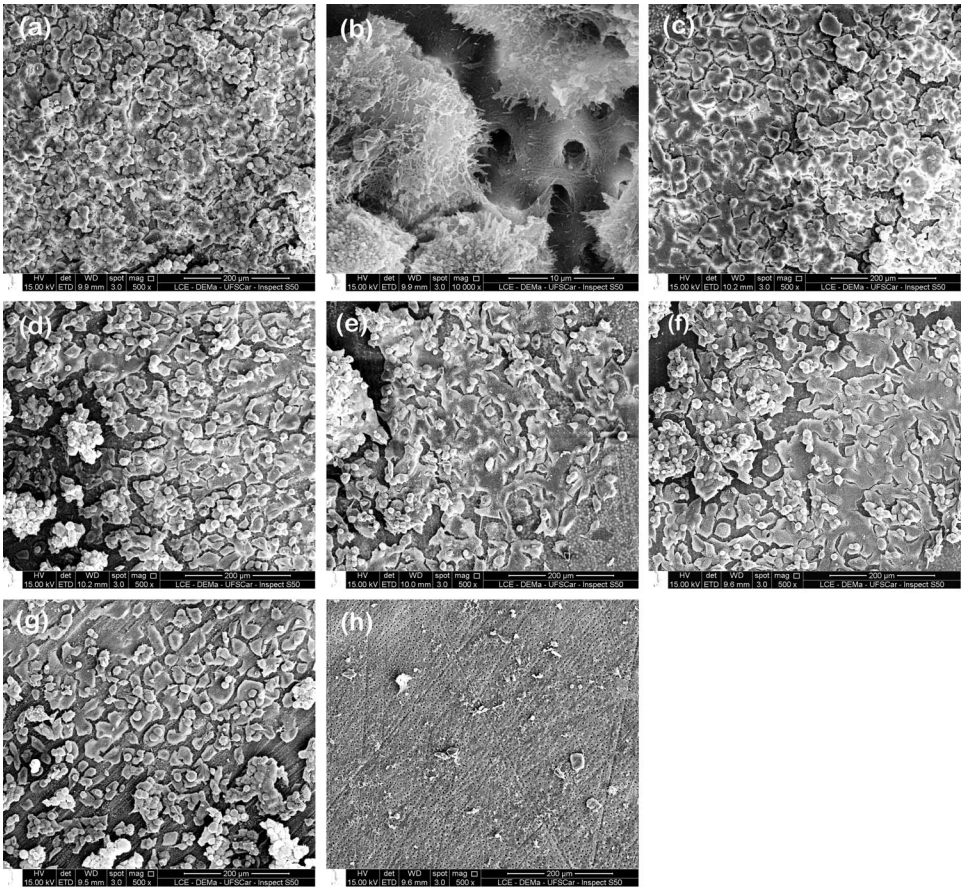

Figure 2. *Composite figure of SEM micrographs representative of negative control (a and b), Sorensen buffer (c), 0.1 M EDC (d), 0.3 M EDC (e), 0.5 M EDC (f), 5% glutaraldehyde (g), and 29% hydrogen peroxide (positive control) (h). SEM 500×. A large number of MDPC-23 cells remained attached to the dentin surface, except in the positive control group (h). In that particular group most cells detached from the dentin, and only residual fragments of dead cell are seen. In (b) it is possible to observe the large number of microextensions of the cells, responsible for the cell fixation to the dentin substrate seen in the background of the image. SEM 10,000×.*

with deionized water did not exert cytotoxic effects on odontoblast-like cell MDPC-23 even when the remaining dentin was as thin as 0.4 mm. These results require acceptance of the tested null hypothesis. In addition, MTT data associated with TP production seem to indicate that GA was able to slightly increase cell metabolism, the opposite of what was expected.

Some critics may wonder why EDC and GA were not cytotoxic when applied topically to dentin, while they have been shown to be cytotoxic when applied directly to cells. The answer lies in the structure and function of dentin as a protective barrier. Dentin protects the pulp from toxic chemicals in at least three ways: 1) the surface area of fluid-filled dentinal tubules only represents 1% of the physical surface area. That is, mineralized matrices occupy 99% of the surface area of superficial dentin. In deep dentin, open dentinal tubules may occupy up to 22% of the area, while mineralized matrix occupy 78% of the physical surface area.[51] Thus, based on diffusional surface area, dentin restricts diffusion. 2) As solutes diffuse down dentinal tubules, they often bind to hydroxyapatite lining the tubules or the collagen fibrils within the lumen. GA has been shown to react with albumin and other plasma proteins present in dentinal fluid to form insoluble precipitates of proteins that tend to decrease dentin permeability.[47,48] 3) The chemical concentration driving diffusivity is the applied concentration that dissipates over distance. That is, the applied concentration rapidly falls the further the solute diffuses into dentin, so that by the time it reaches the other side of the dentin, its concentration has fallen by a 10-, 100-, or 1000-fold measure.[42] Finally, when the dentin surface is rinsed with water to stop inward diffusion by dilution, the solutes in dentin start to diffuse backwards out of the tubules, since there is more solute in the tubules than at the water rinsed surface. All of these processes protect the pulpal

cells from solutes applied to dentin surfaces. If one by-passes the protective effect of dentin by, for instance, applying EDC directly to pulpal soft tissue exposed by a pulp exposure, then pulpal cells would be exposed to much higher EDC concentrations (by 10^3-10^5) than occur following application to intact dentin. This pioneering study in transdentinal cytotoxicity of cross-linking agents showed that the use of these substances in dental practice to enhance resin-dentin bond quality may be safe. Future studies are necessary to better understand the interaction of these agents with pulp tissue and their behavior *in vivo*.

CONCLUSIONS

According to the methodology used in the present investigation, it may be concluded that the treatment of acid-etched dentin with 0.1 M to 0.5 M EDC or 5% GA for 60 seconds does not cause transdentinal cytotoxic effects on cultured odontoblast-like cells.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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