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Use of a specific MMP inhibitor (Galardin) for preservation of hybrid layer

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Abstract

Objective—Dentinal MMPs have been claimed to contribute to the auto-degradation of collagen fibrils within incompletely resin-infiltrated hybrid layers and their inhibition may, therefore, slow the degradation of hybrid layer. This study aimed to determine the contribution of a synthetic MMPs inhibitor (Galardin) to the proteolytic activity of dentinal MMPs and to the morphological and mechanical features of hybrid layers after aging.

Methods—Dentin powder obtained from human molars was treated with Galardin or chlorhexidine digluconate and zymographically analyzed. Microtensile bond strength was also evaluated in extracted human teeth. Exposed dentin was etched with 35% phosphoric acid and specimens were assigned to (1) pre-treatment with Galardin as additional primer for 30s; (2) no pre-treatment. A two-step etch-and-rinse adhesive (Adper Scotchbond 1XT, 3M ESPE) was then applied in accordance with manufacturer's instructions and resin composite build-ups were created. Specimens were immediately tested for their microtensile bond strength or stored in artificial saliva for 12 months prior to being tested. Data were evaluated by two-way ANOVA and Tukey's tests ($\alpha=0.05$). Additional specimens were prepared for interfacial nanoleakage analysis under light microscopy and TEM, quantified by two independent observers and statistically analyzed (χ^2 test, $\alpha=0.05$).

Results—The inhibitory effect of Galardin on dentinal MMPs was confirmed by zymographic analysis, as complete inhibition of both MMP-2 and -9 was observed. The use of Galardin had no effect on immediate bond strength, while it significantly decreased bond degradation after 1 year ($p<0.05$). Interfacial nanoleakage expression after aging revealed reduced silver deposits in galardin-treated specimens compared to controls ($p<0.05$).

Conclusions—This study confirmed that the proteolytic activity of dentinal MMPs was inhibited by the use of Galardin in a therapeutic primer. Galardin also partially preserved the mechanical integrity of the hybrid layer created by a two-step etch-and-rinse adhesive after artificial aging.

Keywords

Galardin; dental bonding systems; hybrid layer; aging; dentin

Introduction

Formation of a perfect resin-infiltrated hybrid layer, composed of collagen fibrils embedded by methacrylate-based resins, has been thought as essential to provide durable and successful adhesion to human dentin. The breakdown of resin-bonded interfaces has been directly related with the loss of stability of the hydrophilic resin components that comprises the hybrid layers [1-3]. Nevertheless, the clinical implications of the integrity of dentin matrices within *in vivo* hybrid layers have been increasingly demonstrated as being fundamental for the loss of longevity of resin-bonded restorations [3-5].

The outstanding resistance of the collagenous dentin matrix against thermal and proteolytic disruption has been attributed to the high degree of intermolecular crosslinking and tight mechanical weave of this specialized connective tissue [6-8]. However, great attention to the potential proteolytic activity of dentin has been raised since complexed and active forms of matrix metalloproteinases (MMPs) were identified in either non-mineralized or mineralized compartments of human dentin matrices [9-15]. MMPs belong to a group of zinc- and calcium-dependent enzymes that have been shown to be able to cleave native collagenous tissues at neutral pH in the metabolism of all connective tissues [16,17].

Dentin matrix has shown to contain at least four MMPs: the stromelysin-1 (MMP-3) [15], the true collagenase (MMP-8) [14] and the gelatinases A and B, (MMP-2 and MMP-9 respectively) [9,13]. These host-derived proteases are thought to play an important role in numerous physiological and pathological processes occurring in dentin, including the degradation of collagen fibrils that are exposed by suboptimally infiltrated dental adhesive systems after acid etching [4,5,12,18].

Collagenolytic/gelatinolytic activity of human dentin matrices [12] has recently shown to be suppressed by a nonspecific protease inhibitor, chlorhexidine digluconate (CHX) [19], at two concentrations: 0.2% (2.2 mM) [20] and 2% (22 mM) [4,5,12,20] and this inhibition has reported to be beneficial in the preservation of the hybrid layer both *in vivo* [4,5,21] and *in vitro* [20,22].

Although the use of CHX as antiproteolytic primer for dentin matrix preservation has shown to be extremely convenient due to its commercial availability as mouthrinse agent (at 0.12 – 0.2%) and cavity disinfectant (at 2%), it is quite reasonable to speculate that specific MMP inhibitors, which normally exert inhibitory activity under extremely low concentrations, may be as or even more effective than CHX in increasing the durability of hybrid layers.

Galardin is a synthetic MMP inhibitor with potent activity against MMP-1, -2, -3 -8 and -9 [23,24] and its action was firstly reported in the 90's by Grobelny *et al* [23]. Galardin has a collagen-like backbone to facilitate binding to the active site of MMPs and a hydroxamate structure (R-CO-NH-OH, where R is an organic residue) which chelates the zinc ion located in the catalytic domain of MMPs [25]. Galardin, also known as GM6001 or Ilomastat, has been used to selectively inhibit MMP-2, -3, -8 and -9 [26,27] that have already been demonstrated to be present in human dentin matrix [9,13,14,15].

Thus, the aim of this study was to test the efficacy of galardin on the morphological and mechanical preservation of aged hybrid layers. It was hypothesized that: 1) the gelatinolytic

activity of phosphoric-acid demineralized dentin is similarly inhibited by CHX and galardin; 2) galardin can preserve the morphological and mechanical integrity of aged hybrid layers.

Materials and Methods

Sixty recently extracted, non-carious human molars were collected after the patients' informed consent had been obtained under a protocol reviewed and approved by the Ethic Committee for Human Studies of the University of Trieste.

Zymographic analysis (Gelatinolytic activity of phosphoric-acid demineralized dentin)

Reagents were purchased from Sigma Chemical (St Louis, MO, USA) unless otherwise specified.

Twenty teeth were selected for this part of the study. Enamel and residual pulp tissue of these teeth were removed and dentin powder was obtained by pulverizing the liquid nitrogen-frozen dentin with a steel mortar/pestle (Reimiller, Reggio Emilia, Italy) in accordance with Mazzoni *et al.* [13]. Five 1-g aliquots of dentin powder were obtained and assigned to one of the following treatments: Group 1: untreated mineralized dentin powder; Group 2: dentin powder partially demineralized with 1% phosphoric acid for 10 min at 4°C; Group 3: dentin powder partially demineralized with 1% phosphoric acid for 10 min at 4°C then incubated with 0.2 mM galardin (water solution) for 30 min; Group 4 and 5: dentin powder partially demineralized with 1% phosphoric for 10 min then treated, respectively, with 2.2 mM (0.2%) and 22 mM (2%) CHX water solution for 30 min. All specimens were then rinsed with 1 mL of distilled water (5 times), then re-suspended for 24 h in 4 mL extraction buffer: 50 mM Tris-HCl pH 6, containing 5 mM CaCl₂, 100 mM NaCl, 0.1% Triton X-100, 0.1% nonionic detergent P-40, 0.1 mM ZnCl₂, 0.02% NaN₃ and EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Germany). Specimens were centrifuged at 14000 rpm (Eppendorf, Minispin Plus, Hamburg, Germany), supernatants were collected and protein content was precipitated with 25% trichloroacetic acid (TCA) at 4°C. TCA precipitates were re-solubilized in loading buffer pH 8.8 containing Trizma and 2% sodium dodecyl sulphate (SDS) in water.

Total protein concentration of mineralized and partially demineralized dentin extracts was determined using the Bradford assay. Proteins were electrophorized under non-reducing conditions on SDS-polyacrylamide gels copolymerized with 2 g/L gelatin (porcine skin). Activation of gelatinase proforms was achieved with 2 mM p-aminophenylmercuric acetate (APMA) for 1 hr at 37°C and then the SDS-polyacrylamide gels were incubated for 24 hrs at 37°C in zymography buffer (CaCl₂, NaCl and tris-HCl, pH 8.0). Gels were stained in 0.2% Coomassie Brilliant Blue R-250 and destained in destaining buffer (50% methanol, 10% acetic acid, 40% water).

Control zymograms were incubated in the presence of 5 mM EDTA and 2 mM 1,10-phenanthroline to inhibit gelatinases.

Microtensile bond strength test

Twenty-eight of the selected teeth were used in this part of the study. Flat surfaces of middle/deep dentin were exposed with a slow speed diamond saw (Micromet, Bologna, Italy). Smear layer-covered dentin surfaces were etched with 35% phosphoric acid for 15 s (etching gel, 3M ESPE), rinsed and surfaces were blot dried according to the wet bonding technique. Specimens were randomly assigned to the following treatments (N=14). Groups 1: acid-etched dentin surfaces were treated with 0.2 mM galardin (water solution) for 30 s, blot-dried and bonded with SB1XT; Group 2: received no pre-treatment before SB1XT

application (control). SBIXT was applied in accordance with manufacturer's instructions and light-cured. Resin composite build-ups were created with Filtek Z250 (3M ESPE, St. Paul, MN, USA).

Resin-dentin sticks with cross-sectional area of approximately 0.9 mm^2 were obtained in accordance with the non-trimming technique [28]. Each stick was measured and recorded for bond strength calculation. Sticks were divided in two equal groups and either stored at 37°C for 24h (T_0) or for 1yr ($T_{1\text{yr}}$) in artificial saliva prepared in accordance with Pashley *et al.* [12], but without protease inhibitors. Sticks were stressed until failure with a simplified universal testing machine at a crosshead speed of 1 mm/min (Bisco Inc., Schaumburg, IL, USA). Failure modes were evaluated as described by Breschi *et al.* [20].

The number of prematurely debonded sticks per each tested group was also recorded, but not included in the statistical analysis since all premature failures occurred during the cutting procedure, which was performed at time zero and did not exceed 3% of the total number of tested specimens. As values were normally distributed (Kolmogorov-Smirnov test), data were analyzed with a two-way ANOVA (tested variables were: presence of galardin, time of storage) and Tukey's post hoc tests. To analyze the effect of galardin on fracture modes, mixed and dentin cohesive failures were combined when performing the statistical analysis. Wilcoxon Signed Ranks Test was used to analyze the differences in failure modes between T_0 and $T_{1\text{yr}}$ for each group, and Kruskal-Wallis test was used to compare the fracture modes between the groups within each time points. Statistical significance was set at $\alpha=0.05$.

Nanoleakage evaluation

The remaining twelve teeth ($N=3/\text{group}$) were prepared and bonded as previously described for nanoleakage evaluation. Resin-bonded specimens were then vertically cut into 1-mm thick slabs to expose the bonded surfaces, which were further submitted to storage in artificial saliva at 37°C for two different times: T_0 (24 hr) and $T_{1\text{yr}}$ (1 year). After storage (24hr or 1yr), the resin-bonded interfaces were immersed in 50 wt% ammoniacal AgNO_3 solution according to the protocol described by Tay *et al.* [1], thoroughly rinsed in distilled water, and immersed in photodeveloping solution. The silver-impregnated specimens were fixed, dehydrated, embedded in epoxy resin (Epon 812; Fluka, Buchs, Switzerland), and processed for light microscopy (LM) in accordance with Saboia *et al.* [29].

Briefly, specimens were fixed on glass slides using cyanoacrylate glue, flattened with 600, 800, 1200, and 2400 grit SiC paper under running water (LS2; Remet) and stained with 0.5% acid fuchsin for 15 min, finally analyzed by light microscopy (Nikon E 800; Nikon, Tokyo, Japan). All interface images were obtained at a magnification of $100\times$ and the amount of silver tracer precipitation along the interface (*i.e.*, the degree of interfacial nanoleakage) was scored on a scale of 0 to 4 by two observers in accordance with Saboia *et al.* [29], *i.e.* interfacial nanoleakage was scored based on the percentage of the adhesive surface showing silver nitrate deposition: 0, no nanoleakage; 1, $< 25\%$ with nanoleakage; 2, $25 \leq 50\%$ with nanoleakage; 3, $50 \leq 75\%$ with nanoleakage; 4, $> 75\%$ with nanoleakage (Table 2). Intra-examiner reliability was assessed by the Kappa test ($K=0.84$). Statistical differences among score groups (*i.e.* number of specimens falling within each score category) were analysed using the χ^2 -test ($p<0.05$).

Additional embedded specimens were processed for nanoleakage analysis under TEM in accordance with Suppa *et al.* [30] and examined under TEM (Philips CM-10, Eindhoven, The Netherlands) operating at 70 kV.

Results

Zymographic analysis

Mineralized dentin powder did not show any enzymatic activity (Group 1, Fig.1, Lane 1). Zymograms of 1% phosphoric acid demineralized dentin extracts (Group 2) showed multiple forms of gelatinolytic enzymes (Fig. 1, Lane 2), with the 66-kDa activity identified as MMP-2 active-form, and fainter bands of 92 kDa, as pro-MMP-9 along with truncated forms. Treatment with 0.2 mM galardin (Fig.1, Lane 3), 2.2 mM or 22mM CHX (Fig. 1, Lane 4 and 5) showed complete inhibition of both MMP-2 and -9 activities.

Control zymograms incubated with 5 mM EDTA and 2 mM 1,10-phenanthroline showed no enzymatic activity (data not shown).

Microtensile bond strength and fracture mode analyses

Mean bond strength obtained at T_0 and T_{1yr} and failure mode distributions are summarized in Table 2. At T_0 , there were no differences between the bond strength of galardin-treated specimens vs control (galardin+SB1XT = 44.1 ± 7.3 MPa; SB1XT control = 41.4 ± 5.9 MPa; $p > 0.05$). A significant decrease in bond strengths was found after 1 year of *in vitro* storage ($T_0 > T_{1yr}$; $p < 0.05$), however while bond strength decreased approximately 27% in the galardin-treated group, the decrease of bond strength was up to 45% in SB1XT-control group (galardin + SB1XT = 32.4 ± 6.6 ; SB1XT control = 22.6 ± 5.4 MPa; $p < 0.05$). Fracture mode analysis did not demonstrate any statistically significant differences between the groups or within the groups at either time points (Table 2).

Interfacial nanoleakage analysis

Data regarding the extent of silver nitrate deposition along the bonded interface performed under LM are shown in Table 2. Although 1 yr of *in vitro* aging in artificial saliva caused increase in interfacial nanoleakage expression ($p < 0.05$), the galardin-treated group showed minor nanoleakage expression compared to the untreated control specimens (Fig. 2; Table 2; $p < 0.05$). TEM correlative analysis of the interfacial nanoleakage expression produced similar results (Fig. 3,4).

Discussion

Complete inhibition of the gelatinolytic activity of phosphoric-acid demineralized dentin was verified in zymograms after treatment of dentin powder with 0.2 mM galardin, 2.2 mM (0.2%) and 22 mM (2%) CHX. No difference in the enzymatic inhibition of demineralized dentin was observed when galardin- and CHX-treated dentin specimens were conjunctly assayed, which supports the acceptance of the first hypothesis of this study. Galardin-treated dentin showed a 27% reduction in bond strength after 1 yr of aging that was significantly less ($p < 0.05$) than the 45% reduction in bond strength seen in untreated controls. Galardin-treatment also substantially reduced the nanoleakage of resin-bonded interfaces at 1 yr as well. Accordingly, these results support the acceptance of the second testing hypothesis.

Nanoleakage involves the diffusion of ammoniacal silver nitrate into water-filled voids or channels in hybrid layers. As the uninhibited endogenous MMP activity resin-infiltrated dentin matrices expresses itself over 1 year of storage, insoluble collagen fibrils are slowly broken down to gelatin. Gelatin peptides are then broken down by gelatinases to smaller peptides and amino acids that elute from the degrading hybrid layers. Their mass is replaced by water. Thus, higher silver uptake equates with the increase in water uptake that follows loss of collagen and resin stability. The increase in nanoleakage is consistent with the reported degradations of collagen fibrils in hybrid layers [4,5,21,31]. The increased

presence of nanoleakage in the adhesive layer after 1 year of storage has previously been reported by Tay *et al.* [32] and by Reis *et al.* [33].

The present study supports the concept that the poor stability of hybrid layers is partly related to the activity of endogenous dentinal MMPs [9,12,14,15,18,34]. The fact that CHX has shown to preserve *in vivo* and *in vitro* hybrid layers is a breakthrough, but it is an indirect demonstration of the contribution of MMPs embedded within the dentin matrix for resin-dentin bonds degradation. In other words, CHX being a broad-spectrum antimicrobial agent and also a non-specific MMP-inhibitor, it might have enhanced the lifetime of tested hybrid layers not solely by controlling the activity of MMPs, but also by inhibiting other dentin matrix enzymes or even by a detergent-like effect than enhanced the impregnation of resin monomers into demineralized dentin. However, since galardin (a specific synthetic MMP-inhibitor) currently demonstrated a similar ability to slow the breakdown of resin-bonded interfaces, as previously showed when using CHX [4,5,20,21], it is now more evident that specific inhibition of dentinal MMPs may be important to increase the life span of adhesive restorations.

Some collagenase/gelatinase inhibitors act by non-specific chelation of cations such as zinc and calcium that are required to maintain the enzymatic function of MMPs. These inhibitors include EDTA [35], tetracyclines [36] and CHX [19]. Nevertheless, advantage can be taken from an agent that specifically inhibits a protein-target, as its inhibitory function is effectively reached at very low concentrations. Generally, the effective concentration of specific MMP-inhibitors is in the nanomolar range. For instance, galardin was previously shown to efficiently inhibit some MMPs with concentrations as low as 0.4 nM for MMP-2 (gelatinase A), 0.5 nM for MMP-3 (stromelysin-1), 3.7 nM for MMP-8 (neutrophil collagenase) and 0.1 nM for MMP-9 (gelatinase B) [26,27].

Previous MMP inhibition studies done with Galardin were done using soluble enzymes. These studies demonstrated that nanomolar concentrations of Galardin were effective at inhibiting MMPs [26,27]. In adhesive dentistry, acid etching both uncovers and activates MMPs that remain bound to collagen. The concentration of Galardin required to completely inhibit the endogenous bound MMPs in dentin may be much higher than that of unbound soluble MMPs.

The similarity between the inhibitory function of 0.2 mM galardin compared with that provided by 2.2 mM (0.2%) and 22 mM (2%) CHX in the zymographic analysis strengthens the concept that the use of specific permits much lower concentrations for enzymatic inhibition. It is notable that by using a concentration approximately 10 to 100 times lower, galardin inhibited the proteolytic activity of demineralized dentin as efficiently as performed by CHX. We speculate that even lower concentrations of other specific MMP inhibitors could lead to complete inhibition of hybrid layer degradation. While galardin is an effective inhibitor of pure soluble MMPs even at lower concentrations than the currently tested one [26,27], we decided to use a galardin saturated solution (0.2 mM if water is used as solvent) to challenge simultaneously its effect on the proteolytic activity of dentin and its possible interference on the formation of the hybrid layer. Since the results demonstrated that 0.2 mM galardin did not affect the immediate bond strength and nanoleakage, while it significantly reduced decreases bond strength over time (1yr-result), this study supports the hypothesis that galardin does not interfere with the hybrid layer formation when a two-step etch-and-rinse adhesive is used.

It is noticeable that galardin-treatment slowed the decline of bond strength and reduced the amount of nanoleakage, but it did not completely block these phenomena (Table 2; Figure 2,3). The observed decline in the bond strength of galardin-treated specimens can be related

with the loss of integrity of resinous components within hybrid layers due to polymer swelling and resin leaching that occurs after water/oral fluid sorption [37,38]. The leaching effect may be especially strong in *in vitro* microtensile testing, as comparable decrease in bond strength was observed with CHX *in vitro* [22], while the loss of bond strength was completely inhibited *in vivo* [5]. In any case, to maximize the stability of such bonded interfaces, efforts should be made to combine the use of antiproteolytic agents with the use of relatively hydrophobic monomers to infiltrate the dentin matrix.

The results of the current study indicate that galardin as an inhibitor of dentinal MMPs may be a clinically relevant way to improve hybrid layer long-term stability. Galardin may be as stable as CHX in hybrid layers. Perhaps galardin should be delivered as an ethanol solution at higher concentrations. In ophthalmology, corneal ulcerations induced by herpetic or alkaline solutions activate corneal MMPs and continue to grow unless inhibited. The use of prolonged topical treatment (30 days) of corneal ulcerations by 1 mM galardin caused no toxic effects [39]. Our use of topical galardin to stabilize the collagen fibrils of hybrid layers is an analogous example of how MMP-inhibitors used for many years in medicine, can be utilized in dentistry.

Despite the fact that the role of endogenous enzymes in the degradation of the hybrid layer is not fully elucidated, this study clarifies the importance of their specific inhibition to preserve the hybrid layer.

Previously published work emphasized the degradation of dental resins as being responsible for reductions in bond strength over time. However, the *in vivo* study by Carrilho *et al.* [5] showed that 2% CHX prevented decreases in bond strength for 14 months and prevented degradation of collagen fibrils in hybrid layers. That study clearly identified that preservation of collagen, not resin preserved bond strength for SB1XT. The current study further supports the Carrilho *et al.* [5] report by showing that specific MMP-inhibitors can also preserve bond strength over time. These results indicate that a paradigm shift has occurred in adhesive dentistry. That is, that preservation of resin-bonded interfaces requires prevention of degradation of collagen fibrils in hybrid layers.

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Figure 1.

Gelatin zymogram of MMPs from dentin extracts after sonication, TCA precipitation, resuspension and activation with 2mM APMA for 1 hour. Molecular masses, expressed in kDa, are reported in Std lane. *Lane 1*: Absence of any gelatinolytic activities in proteins extracted from mineralized dentin powder; *Lane 2*: identification of MMP-2 and -9 isoforms in phosphoric acid-treated dentin; *Lane 3*: Phosphoric acid-demineralized dentin incubated with Galardin revealed inhibition of all MMPs isoforms; *Lanes 4 and 5*: incubation with 0.2 or 2% CHX respectively, produced complete inhibition of all forms of MMP-2 and -9 activity in phosphoric acid demineralized dentin.

Figure 2.

Light microscopy images of bonded interfaces created by Adper Scotchbond 1XT stored for 1 yr in artificial saliva at 37°C. The semi-thin sections reveal that the use of Galardin as therapeutic primer significantly reduced the amount of silver nitrate at the interface (Fig. 2a; pointers), compared to extensive nanoleakage formation that can be seen along the hybrid layer on control specimens (Fig. 2b; pointers).

Figure 3.

TEM image obtained combining several micrographs of a representative specimen treated with Galardin for 30s, then bonded with SB1XT and stored for 1 yr in artificial saliva at 37°C. The adhesive (A) interface revealed few scattered clusters (pointers) of silver nanoleakage within the hybrid layers (HL). MD = mineralized dentin; T = dentinal tubules; A = filled adhesive. Bar = 2 μ m.

Figure 4.

TEM image obtained combining several micrographs of a representative control specimen bonded with SB1XT and stored for 1 yr in artificial saliva at 37°C. The adhesive interface reveals extensive interfacial silver nanoleakage due to large clusters of silver deposits (pointers) within the collagen fibrils of the hybrid layer (HL). Abbreviations are same as in Fig. 2. Bar = 2 μ m.

Table 1
Composition of Adper Scotchbond 1 XT and mode of application

Adhesive	Composition	Mode of application
Adper Scotchbond 1XT	<p><i>Etching:</i></p> <p>35% phosphoric acid</p> <p><i>Primer/Bond:</i></p> <p>2-hydroxyethylmethacrylate (HEMA)</p> <p>Polyalkenoic acid copolymer</p> <p>Bis-phenol A diglycidylmethacrylate (Bis- GMA)</p> <p>Water</p> <p>Ethanol</p> <p>Silica particles</p>	<p>etching 15 s</p> <p>water rinsing 30 s</p> <p>dentine blot drying</p> <p>adhesive application</p>

Table 2

Bond strengths of 0.04% Galardin (GL)-treated specimens bonded with Adper Scotchbond 1XT vs control that were tested immediately (T_0) or after 1 year (T_{1yr}) of aging in artificial saliva. Distribution of failure mode (in %) among tested groups in the different periods of analysis and nanoleakage expression at light microscopy are also reported. A: adhesive; CD: cohesive failure in dentin; CC: cohesive failure in resin composite; M: mixed failure, as described by Breschi *et al.*, 2008b.

Bonding strategy	Bond strength	Failure Mode (%)			
		A	CD	CC	M
GL + SB1XT	immediate	30 ^A	5 ^B	20 ^A	55 ^C
	1yr	26 ^A	7 ^B	18 ^A	49 ^C
SB1XT control	immediate	20 ^A	7 ^B	28 ^A	45 ^C
	1yr	24 ^A	5 ^B	19 ^A	52 ^C

Values are mean $\pm$ SD (number of teeth) in MPa [number of premature failed sticks/number of intact sticks tested]. Groups identified by different lower case letters are significantly different ($p < 0.05$). Groups identified by the same uppercase letters are not significantly different ($p > 0.05$).

Table 3

Distribution of nanoleakage in Galardin (GL-) treated specimen and controls 24 hours and 1 year after composite adhesion as described in Saboia *et al.*, 2008. Numbers in Table indicate the percentage of samples with respective nanoleakage (from 0 to >75% of adhesive joint) as observed with 100x magnification in light microscope. The respective bars demonstrate significantly smaller percentage (12%) of marked (>50%) nanoleakage in GL-treated samples compared to controls (59%) after 1 year storage in vitro in artificial saliva.