Does etching time affect the \textit{in vitro} performance of a sealant material?

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This \textit{in vitro} study evaluated the shear bond strength (SBS), failure mode and microleakage of fissure sealing materials in relation to different etching times on aprismatic and prismatic enamel. Ninety-six healthy human third molars were randomly allocated to the following groups: 1) prismatic or aprismatic enamel; 2) etching: 15, 30, 45, and 60 s. After 5,000-fold thermocycling SBS, failure mode and microleakage were measured. Statistical evaluation included Mann-Whitney-U-test and linear regression analysis. In the aprismatic enamel group, an increasing etching time resulted in higher SBS. The linear regression model revealed that 60 s of etching time led to a significant increase in SBS. Microleakage was found to be low in all test groups. This study indicated that 60 s of etching time showed to a significantly better SBS. When considering the small differences of SBS, failure modes and microleakage between 30 and 60 s etching time, 30 s acid etching seems to also be justifiable.

\textbf{Keywords:} Pit and fissure sealing, Enamel pretreatment, Acid etching, Shear bond strength, Microleakage testing

\section*{INTRODUCTION}

Sealing pits and fissures is an effective caries-preventive measure\textsuperscript{11} and is mostly indicated for caries-active children today. When considering the clinical workflow, it is evident that acid etching is an important pretreatment step to guarantee long-lasting bonding of the sealant material on the enamel. The shortening of acid conditioning has been discussed repeatedly since the introduction of pit and fissure sealants to simplify the treatment and reduce the chair time, particularly in children\textsuperscript{3-5}. It is surprising that to the best of our knowledge, only limited data from comparative clinical studies\textsuperscript{5-9} exist regarding the influence on longevity. Only one study was conducted, in which Duggal \textit{et al.}\textsuperscript{5} found 40.0, 50.9, 41.8 and 43.6\% intact sealants after 12 months when etching the enamel for 15, 30, 45 and 60 s, respectively. When comparing these data with longevity results from the overwhelming majority of clinical studies, retention rates of approximately 80\% can be expected after two years when using a minimum etching time of 30 s\textsuperscript{6}. The documented results seem not to be fully plausible and should therefore be re-evaluated. Independent of clinical experience, few \textit{in vitro} studies have investigated the influence of the length of acid conditioning on the \textit{in vitro} performance of sealant materials, \textit{e.g.}, bond strength or microleakage\textsuperscript{8-10}. With respect to the existing knowledge gaps, it seems difficult to reach final conclusions for daily dental practice. Therefore, this comparative \textit{in vitro} study aimed to contribute to this discussion and assess the shear bond strength (SBS) and microleakage of a sealant material under the inclusion of different commonly recommended acid etching times, namely, 15, 30, 45, and 60 s with permanent tooth material. The null hypothesis of this study was that there would be no difference in SBS and microleakage performance between the different etching times and enamel preparation.

\section*{MATERIALS AND METHODS}

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee.

\textbf{Sample preparation}

Ninety-six healthy, caries-free, extracted human third molars were used in this investigation for testing SBS. All teeth were free of any developmental disorders, fillings, and fissure sealants and showed complete root development. Sclerotic teeth from elderlies were excluded as well. After extraction, the teeth were stored in sodium azide solution (0.2\%). Prior to use, the teeth were washed thoroughly under running water to eliminate the remaining traces of storage solution. The roots were sectioned 1 mm apically to the cementoenamel junction, and the crowns were further sectioned into 3 parts (mesial, buccal, lingual) with a diamond disc (Dental Diamond Disc, H 340-F-300, Horico, Berlin, Germany). The distal surfaces were excluded from the sample collection due to the findings from a previously published article\textsuperscript{11}. This process resulted in 288 tooth surfaces, which were randomly assigned to 8 study groups ($n$=36; 12 samples from the mesial, buccal, and lingual surfaces). Randomization was coded such that only one surface from each tooth was assigned to a group (Fig. 1). All samples were numbered according to the randomization table.

All tooth surfaces were embedded in cold-curing methyl methacrylate resin (Technovit 4004, Heraeus
Kulzer, Wehrheim, Germany) by means of a 15-hole mould (Ultradent Products, South Jordan, UT, USA). Each flat, superficial enamel surface was situated horizontally in the embedding material, producing cylinders measuring 1 inch in diameter by 1 inch in length. To ensure that the bottom surface of the specimen was parallel to the top surface, a mounted grinding mandrel (Ultradent Products) on a model trimmer’s working table (HSS88, Wassermann, Hamburg, Germany) was used. After embedding, all tooth surfaces were carefully cleaned and rinsed with water spray. The SBS was compared between different etching times (15, 30, 45 and 60 s) on aprismatic (unground) and prismatic (ground) enamel.

Enamel preparation
In the group with prismatic enamel, standard surfaces were prepared as follows: To ensure that the ground enamel surface of the tooth was parallel to the bottom surface, a mounted grinding mandrel on a model trimmer’s working table was used initially to produce a flat, parallel grinding area, which was sufficient to place a sealant button to obtain a standardized horizontal plane for SBS testing. Next, 120-grit silicon carbide abrasive paper (Leco, St. Joseph, MI, USA) under running water on a grinding machine (VP100, Leco) and 400-grit paper (Leco) was applied until the surface was even and smooth. In the aprismatic group, which simulated the real clinical situation of fissure sealing, the natural enamel surface of each specimen was not ground.

Placing the fissure sealant material
In vitro application of the sealant was strictly performed according to the manufacturer’s clinical recommendations. The tooth surface was initially cleaned with a fluoride-free polishing paste (Zircate Prophy Paste, Dentsply De Trey, York, PA, USA) and rinsed with a water spray and dried with water- and oil-free air. After checking the cleanliness of each enamel surface under a stereomicroscope (Stemi SV11, Carl Zeiss, Jena, Germany), an etching procedure with 37% phosphoric acid gel (Total Etch, Lot W98952, Ivoclar Vivadent, Schaan, Liechtenstein) was performed for 15, 30, 45 or 60 s. The tooth surface was then rinsed with a water spray for 20 s and dried with pressurized air for 5 s until a chalky-white enamel surface was visible. The teeth were then inserted into the bonding clamp, and the cylindrical plastic mould (Button Mold Insert, ISO 29022, Ultradent Products) was adapted to achieve a gap-free fit on the enamel surface. The sealant (Helioseal F, Lot X08122, Ivoclar Vivadent) was then applied in two steps. Each layer (~2 mm) was light cured for 20 s with a light-curing unit (Bluephase Style, 1,200 mW/cm², wavelength 385 to 515 nm, Ivoclar Vivadent). To prevent uncontrolled leakage of the fissure sealant onto the enamel, a light-cured resin barrier (OpalDam, Ultradent Products) was applied around the perimeter of the bonding mould before applying the fissure sealant. After light curing, the resin barrier and bonding mould were carefully removed. The procedure rendered a sealant cylinder 2.37 mm in diameter perpendicular to the enamel surface, as required by ISO 29022:2013.

Sample ageing
The samples were aged according to our previous protocols: First, one-day storage in distilled water at 37°C in a thermal oven (Modell 400, Memmert, Schwabach, Germany) followed by thermocycling (Haake W15, Thermo Haake, Karlsruhe, Germany) between 5°C (±2°C) and 55°C (±2°C) for 5,000 cycles, with a dwell time of 30 s and a transfer time of 5 s.

Notched-edge SBS testing and failure mode analysis
The notched-edge (ISO standard) SBS test was performed using a universal testing machine (MCE 2000ST, Quicktest Prüfpartner, Langenfeld, Germany). First, the samples were placed in a metal sample holder (Test Base Clamp, ISO 29022, Ultradent Products) with the occlusal surface facing down. A notched-edge shear fixture with a semicircular moulded shear blade (Notched-edge Shear Blade, ISO 29022, Ultradent Products) was mounted on the universal testing machine and placed over the sealant cylinder on the aligned specimen. The notch sedge shear blade had to be positioned exactly over the cylinder and force fitted without premature contact to ensure that the load was applied directly to
the cylinder. A constant crosshead speed of 0.5 mm/min was applied until the material failed. The maximum force (N) at failure was recorded. Considering the bonded area of the fissure sealant on the tooth surface, the SBS was calculated in MPa.

All samples were examined for failure modes under a stereomicroscope at 20-fold magnification. The failure mode was described as follows: 1. adhesive, 2. cohesive within the material, 3. mixed (adhesive and cohesive within the material), and 4. enamel failure [13].

Microleakage testing

Five human third molars were assigned to each of the eight groups. All teeth were stored and cleaned as previously described. Each tooth was taken as a whole, and fissure sealing on the prismless enamel of the occlusal fissure pattern was performed in strict accordance with the manufacturer’s instructions (see above). For the group with prismatic enamel, the superficial enamel in the areas of the central groove and supplemental groove as well as the involved area of each cusp were removed by a flame-shaped finishing diamond bur (FG 5236, Intensive, Montagnola, Switzerland). All samples were stored in distilled water at 37°C for 24 h in a thermal oven and aged in a thermocycling bath as described above. After thermocycling, the root surfaces were isolated with tacky wax (Boxing Wax Sticks, Kerr, Romulus, MI, USA). Afterwards, the entire tooth surface was covered with two layers of nail varnish, except the area within 1 mm of the fissure seal. The varnish was applied to avoid dye penetration to other parts of the tooth. The samples were then immersed in 0.5% methylene blue solution (Methylene Blue Extra Pure, Merck, Darmstadt, Germany) for 24 h at 37°C. All samples were rinsed with water, and the roots were sectioned off 1 mm below the cementoenamel junction with a diamond disc. The tooth crowns were then fully embedded in cold-curing methyl methacrylate resin. This treatment resulted in a rectangular block of approximately 2.5×1.2×0.8 cm for each tooth. The blocks were fixed in a sectioning saw (Isomet Low Speed Saw, Buehler, Lake Bluff, IL, USA) with a diamond blade (Diamond Blade, Leco), and the crowns were sectioned in the buccolingual direction into at least 5 slices, each with a thickness of 1 mm. The front and back of each slice were inspected, resulting in at least 10 available section sides per tooth. The side analysis was performed using a stereomicroscope with a 20-fold magnification. Every side was photographed with a digital single lens reflex camera. The following picture analysis methodically separated all sides without dye penetration and then collected all section sides with dye penetration. Additionally, quality losses, such as defects in sealant materials, were recorded. If dye penetration was present, each side was quantitatively measured in relation to the total length of the interface between the enamel and the sealant. All measurements were performed with the imaging software ImageJ (version 1.52, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The percentage of microleakage was calculated. Microleakage was ruled out for dye penetration through enamel, dentine or fissure sealant cracks or along the cementoenamel junction.

Statistical analysis

Descriptive and explorative data analysis was performed using Stata/SE 14.2 (StataCorp 2015, College Station, TX, USA). Descriptive statistics for SBS and microleakage for each group were calculated. Pairwise comparisons of microleakage values between different etching times (e.g., 15 s aprismatic enamel vs. 30 s aprismatic enamel, 15 s prismatic enamel vs. 30 s prismatic enamel, etc.) were performed using the Mann-Whitney U test. Linear regression analysis was performed to compare the results from SBS testing. The model included etching time (15, 30, 45 and 60 s), type of enamel preparation (apismatic and prismatic) and tooth surface (mesial, buccal and lingual), individually. Although the SBS data were not normally distributed, linear regression was performed as the residuals were normally distributed. For the sake of consistency with the results from the linear regression, all descriptive values are presented as the mean and standard deviation or as percentages. A significance of α=0.05 (two-tailed test) and a 95% confidence level were used for all analyses.

RESULTS

The investigation of the SBS on aprismatic enamel revealed that longer acid etching tended to result in slightly higher fracture resistance, but the difference was not found to be significant (Table 1). When comparing the results for 15 and 60 s etching times, there was an increase from 14.0 to 16.1 MPa. In principle, the same finding was also registered for prismatic enamel. Here, a significant difference between 15 and 60 s as well as between 30 and 60 s acid etching times in the prismatic group was detected (Table 1). There was no significant difference between the tooth surfaces and the type of enamel preparation.

The results for the failure mode analysis (Table 2) revealed that adhesive failure was the most predominant type of failure among all groups, ranging from 75.0 to 94.4%, followed by mixed failures, ranging between 5.6 and 22.2%. Cohesive failures and enamel failures were rarely observed among the groups (Table 2). In general, no serious differences existed between the aprismatic enamel group and prismatic enamel group.

The results from the simple linear regression (Table 3) show that only etching time had a significant influence on the SBS, while the type of enamel preparation or tooth surface played an insignificant role. The estimate from the regression analysis resulted in 13.9 MPa for the reference group (15 s etching time), whereas a 60 s etching time had a positive effect by significantly increasing the SBS by 2.3 MPa. On the other hand, 30 or 45 s etching times did not have any influence on the SBS (Table 3).

The mean values for microleakage were found to be low throughout all groups (Table 4), and no significant difference was detected. However, it should be noted that
Table 1  SBS relative to the 15, 30, 45, and 60 s etching times, type of enamel preparation and tooth surface. Comparative statistical comparisons were made between all etching times in the groups with aprismatic and prismatic enamel, as well as between the corresponding etching times in aprismatic and prismatic enamel.

| Tested tooth surface (n=36) | SBS (mean (SD)) | 15 s | 30 s | 45 s | 60 s | 15 s | 30 s | 45 s | 60 s |
|----------------------------|-----------------|------|------|------|------|------|------|------|------|------|
| Aprismatic enamel | 14.0 (5.6) | 15.4 (6.1) | 14.9 (5.0) | 16.1 (5.4) | 13.8 (4.7)^a | 13.5 (5.6)^b | 15.5 (5.7) | 16.3 (5.9)^a,b |
| Prismatic enamel | 13.5 (5.6) | 15.5 (5.7) | 16.3 (5.9) | 17.1 (6.4) |

^a Pairwise comparison between 15 vs. 60 s acid etching within the prismatic enamel group was found to be statistically significant using a Mann-Whitney U test (p-value: 0.04).

^b Pairwise comparison between 30 vs. 60 s acid etching within the prismatic enamel group was found to be statistically significant using a Mann-Whitney U test (p-value: 0.04).

Table 2  Failure mode analysis relative to the chosen type of enamel preparation and used etching times

| Failure mode analysis N (%) | Aprismatic enamel | Prismatic enamel |
|----------------------------|-----------------|-----------------|
| Notched-edge method (n=36) | 36 (100.0) | 36 (100.0) | 36 (100.0) | 36 (100.0) | 36 (100.0) |
| Adhesive failure | 33 (91.7) | 32 (88.9) | 31 (86.1) | 27 (75.0) | 32 (88.9) | 32 (88.9) | 34 (94.4) | 34 (94.4) |
| Cohesive failure | — | — | — | — | — | — | — | — |
| Mixed failure | 3 (8.3) | 3 (8.3) | 5 (13.9) | 8 (22.2) | 4 (11.1) | 4 (11.1) | 2 (5.6) | 2 (5.6) |
| Enamel failure | — | 1 (2.8) | — | 1 (2.8) | — | — | — | — |

Table 3  The linear logistic regression analysis included etching time (15, 30, 45 and 60 s), type of enamel preparation (aprismatic and prismatic) and tooth surface (mesial, buccal and lingual), individually

| Variable | Simple linear regression analysis |
|----------|----------------------------------|
|          | Estimate (95% CI) for the SBS in MPa | p-value |
| Etching time | 15 s (reference) | 13.90 (12.63; 15.18) | <0.001 |
|            | 30 s                | 0.53 (−1.27; 2.34)   | 0.562 |
|            | 45 s                | 1.26 (−0.55; 3.06)   | 0.171 |
|            | 60 s                | 2.32 (0.51; 4.12)    | 0.012 |
| Type of enamel preparation | Prismatic (reference) | 14.76 (13.85; 15.67) | <0.001 |
|            | Aprismatic          | 0.32 (−0.96; 1.61)   | 0.619 |
| Tooth surface | Mesial (reference) | 14.65 (13.54; 15.76) | <0.001 |
|            | Buccal              | 0.64 (−0.93; 2.21)   | 0.423 |
|            | Lingual             | 0.20 (−1.38; 1.78)   | 0.804 |

*a Taken as a reference in the simple linear regression analysis, indicating a significant influence at p<0.001.

Discussion

This comparative in vitro study investigated the influence of the etching time prior to fissure sealing in...
Second, a slight, mostly insignificant increase in SBS was observed for groups with longer etching times. Pairwise comparative statistical analyses (Mann-Whitney U tests) also revealed that a significant increase in SBS was registered between 15 s/60 s and 30 s/60 s etching times when samples of prismatic enamel were used; all other systematically performed comparisons remained insignificant. Next, when considering the available SBS data between aprismatic and prismatic enamel, it can be concluded that there were no differences between those groups under the same etching time in the use laboratory set-up. This fact describes the conflict between the situation in clinical practice (aprismatic enamel) and compliance with the latest recommendations for laboratory testing\(^\text{12}\) (prismatic enamel), thereby justifying the inclusion of both conditions in one study.

With respect to the results from the linear regression model—which aimed to consider all available variables in one statistical model to describe the possible influence on SBS—it was found that only the parameter of 60 s etching time was statistically significant. All other variables, e.g., 15, 30 and 45 s etching times, type of enamel preparation, and tooth surfaces remained insignificant. These explorative statistical data are basically in line with the previously discussed descriptive data and led to the conclusion that the initially formulated hypothesis—that there is no difference between the different etching times before fissure sealing in terms of SBS—has to be rejected. This finding is, however, basically in line with earlier studies and needs to be further discussed. Here, the study by Holtan et al.\(^\text{13}\) supports our findings for prismatic enamel, showing that compared with 15 s etching time, an etching time of 60 s significantly improved the SBS. On the other hand, the results of the present study do not fully concur with findings from other previously published publications\(^\text{14-16}\), where it was documented that compared with a shorter acid etching time (15 s\(^\text{14,15}\), 20 s\(^\text{16}\)) on prismatic enamel, a longer etching time (60 s\(^\text{14-16}\)) produced a non-significant but still higher SBS. In the case of aprismatic enamel, only one study by Tandon et al.\(^\text{9}\) investigated 15, 30, 60 and 120 s etching times in relation to SBS and observed an increasing SBS with increasing etching time (which was not statistically investigated). When considering the information from available in vitro studies, it

| Number of teeth (N) | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
|---------------------|---|---|---|---|---|---|---|---|
| Number of all available tooth slides with fissure sealants N (%) | (100.0) | (100.0) | (100.0) | (100.0) | (100.0) | (100.0) | (100.0) | (100.0) |
| Slices with no dye penetration N (%) | 44 | 45 | 42 | 46 | 49 | 45 | 50 | 48 |
| Slices with dye penetration along the sealant margin N (%) | 3 | 2 | 1 | 2 | 1 | 2 | 2 | 3 |
| Slices with dye penetration into the sealant material N (%) | — | (4.0) | (4.0) | (2.0) | (2.0) | (2.0) | (2.0) | (2.0) |
| Bubble, with dye penetration N (%) | 1 | 1 | 2 | 1 | — | — | — | — |
| Bubble, without dye penetration N (%) | 4 | 2 | 3 | 2 | — | — | — | — |
| Sides with any quality loss N (%) | 8 | 4 | 8 | 5 | 2 | 4 | 5 | 5 |
| Mean microleakage in % (SD) | 3 | 0 | 1.3 | 0.6 | 0 | 0.7 | 0 | 2.6 |
| Minimum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Maximum | 72.8 | 0 | 33.6 | 23.5 | 0 | 17.8 | 61.4 | 60.6 |

Table 4 Microleakage of the tested sealants following 5,000 cycles of thermocycling

Microleakage (in%) was compared within aprismatic and prismatic enamel groups individually based on different etching times (e.g., 15 s on aprismatic enamel vs. 30 s on aprismatic enamel, 15 s on prismatic enamel vs. 30 s on prismatic enamel, and so on), but none of the comparisons showed a statistically significant difference.
might be concluded that longer acid etching times improved the SBS in comparison to that with shorter acid etching times on prismatic enamel, independently of the preparation of the enamel. With respect to the low numbers of samples in each group (n=30\(^2\), n=10\(^3\), n=10\(^4\), n=6\(^5\)), the results from simple (pairwise) statistical comparisons could explain (non-) significant differences and should therefore not be overrated.

When interpreting the results from the failure mode analysis, it can be pointed out that the data are also in line with the previous investigations\(^1\) to \(^4\). In general, adhesive failures are the most common, and cohesive and mixed failures are rare (Table 2) which is basically in line to previous investigations\(^1\) to \(^4\). Interestingly, there was a minor trend of more mixed failures when etching aprismatic enamel for 60 s. On the one hand, this finding may indicate an increase in adhesive performance; on the other hand, the results should not be overrated. In the case of prismatic enamel, a reversed trend was observed.

The dye penetration test/microleakage seems to be the preferable method to evaluate material variants in the laboratory and to gain safety before clinical trials begin\(^5\). While there were many studies assessing microleakage of pit and fissure sealants\(^6\) to \(^8\), to our knowledge no study was available regarding the microleakage of sealants in relation to different etching times. When analyzing the present data, it can be concluded that the mean values obtained were generally low, which is also consistent with most recently published data on resin-based fissure sealants\(^9\). Nevertheless, the present investigation recorded another non-significant trend in aprismatic enamel: In our study, it was observed that 60 s etching resulted in a lower dye penetration compared to that with 15 s etching (0.6% vs. 3.0%). Again, in the case of prismatic enamel, an opposite trend was observed. The descending performance of microleakage in prismatic enamel might be a sign of over-etching in the case of the 60 s etching time\(^10\) to \(^27\).

The methodological strengths of the present study should be seen in the usage of an equal number of 36 samples in each group, which was randomly allocated to avoid potential sampling bias. To simulate the clinical situation of fissure sealing, we used aprismatic enamel in addition to prismatic enamel because the ground prismatic surface would create a standardized, flat enamel surface\(^2\), which is ideal for bonding but does not reflect the clinical situation. In this present study, the distal surfaces from tooth samples were not included due to the findings from a previous study, in which it was revealed that distal surfaces significantly influenced the SBS\(^3\). All tested groups were subjected to 5,000 cycles of thermocycling as an alteration process, which was recommended as a standard ageing method\(^4\). Due to the simulation of the effect of thermal stress, which is usually encountered in the oral cavity, thermocycling might provide a long-term perspective on the longevity of the investigated sealant material. Furthermore, the notch-edge shearing technique was used in this study. This is a force-fitting technique and was recommended as a standardized method according to the International Organization for Standardization\(^5\). A limitation of this study might be the analysis of the SBS alone without considering the (micro)tensile bond strength test or other mechanical test methods\(^6\) to \(^8\) and that we did not (re)investigate morphological changes on enamel after acid etching\(^9\) to \(^20\). Another limitation would be that all microleakage tests were performed on prismatic enamel only. This was justified due to the clinical relevance and that the placement of sealants on aprismatic enamel is commonly not indicated.

CONCLUSION

When considering the SBS results from this in vitro study, it should be noted that only small differences between all tested groups existed. However, the descriptive data indicate that an increasing time of acid etching resulted in slightly higher SBS, and the linear regression analysis also revealed a significant advantage of 60 s acid etching. Furthermore, the trend of more mixed fracture failures and less microleakage on aprismatic enamel support the recommendation of 60 s acid etching before sealant application. Otherwise, when emphasizing the small test differences between 30 and 60 s acid etching, it can be argued that an application time of 30 s may provide acceptable clinical results. With respect to the limited clinical data\(^9\), more research seems to be needed in the future.

CONFLICTS OF INTEREST

All authors have no conflict of interest to report.

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