A new method: measurement of microleakage volume using human, dog and bovine permanent teeth

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This study investigates the feasibility of a different new approach to determining the microleakage volume associated with dental restorations (Class V cavity restorated with glass ionomer cement + high copper amalgam) and the relative marginal adaptation deficiency of dog, bovine and human permanent teeth in *in vitro* conditions. Also researched is the appropriateness of using dog and bovine teeth in *in vitro* studies rather than human teeth. Our method utilizes the molecular adsorption characteristics of methylene blue. Within the framework of this study, 60 permanent teeth (20 human, 20 dogs and 20 bovine) were used.

These groups were evaluated statistically, of which indicated no statistically significant differences (p > 0.05). It was also concluded that this preliminary investigation showed that the new microleakage volume measurement method may be a valuable new technique for the in vitro study of microleakage dynamics around dental restorations.

One of the most important problems of restorative dentistry today is the failure of restorative materials to completely bond to enamel and dentin, causing microleakage. Microleakage has been defined as the passage of ions, molecules, fluids or bacteria between a cavity wall and the applied restorative material. Microleakage has been

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Table 1. Dye penetration scores in all groups (p > 0.05).

Amalgam + GIC Specimens	Group 1 (Human Teeth)	Group 2 (Dog Teeth)	Group 3 (Bovine Teeth)
N = 10 N	Dye penetration Score	Dye penetration Score	Dye penetration Score
1	3	3	3
2	3	2	2
3	2	1	1
4	1	1	1
5	1	1	1
6	1	1	1
7	1	1	1
8	1	0	1
9	1	0	0
10	0	0	0

reported as the cause of hypersensitivity of restored teeth, discoloration at the margins of cavities and restorations, recurrent caries, pulp inflammation and failure of endodontic treatment (Tjan and Tan, 1991; Taylor and Lynch, 1992; Yavuz and Aydın, 2005).

Microleakage is determined today by many *in vitro* techniques with or without thermal cycling, such as staining; scanning electron microscope; bacterial activity; decay; air pressure; chemical agents; markers; neutron activation analysis; radioisotope; ionization; autoradiography and reversible radioactive adsorption. (Tjan and Tan, 1991; Taylor and Lynch, 1992; Sano et al. 1995; Yavuz and Atakul, 2000; Yavuz and Atakul, 2001a; Yavuz and Atakul, 2001b; Yavuz and Aydin, 2005).

The aim of some researchers is to develop an *in vitro* model to replicate microleakage at a tooth/restoration interface (Iwami et al. 2000; Matharu et al. 2001; Yavuz et al. 2003; Yavuz and Aydin, 2005).

The significant differences between these models and materials suggest that an ideal method for the determination of microleakage has not yet been established.

The aim of this study was to develop valuable an *in vitro* model to determine the microleakage volume and ability to use dog and bovine teeth instead of human teeth in *in vitro* studies.

MATERIALS AND METHODS

Sixty recently extracted teeth were selected by Binocular Stereo Microscope (Olympus Co., Japan) for this study; 20 human permanent premolars, 20 bovine permanent incisors and 20 dog permanent canines were used.

Bovine teeth were obtained from the Department of Anatomy of Veterinary Medicine Faculty, dog teeth obtained by the doctor's degree thesis study with the subject of "Lowering of hypertension by nitroglycerin and niprus treatment on the dogs on which pulmonary hypertension is created through pulmonary legation and comparison of its isotonic effect of 7.5% NaCl and 0.9% on vital parameters", and human teeth were obtained from the Department of Maxillo-Facial Surgery of Dental Faculty (Figure 1).

The twenty teeth for each species were randomly divided into two groups (30 teeth per group). One group was used for dye penetration (control group), other group used to study microleakage volume. Before the cavity preparation and restorative procedure, all teeth were cleaned. Class V cavity prepared on the buccal surface of each tooth. The cavity has had enamel and dentin margins. The cavities had a mesio-distal width of 3 mm, an occluso-cervical length of 2 mm and a depth of 1.5 mm.

Table 2. Microleakage volumes in all groups (p > 005).

Amalgam + GIC Specimens	Group 1 (Human Teeth)	Group 2 (Bovine Teeth)	Group 3 (Dog Teeth)
N = 10 N	Volume mm^3/tooth(10^{-3})	Volume mm^3/tooth(10^{-3})	Volume mm^3/tooth(10^{-3})
1	1.91	1.93	1.97
2	2.10	2.06	1.98
3	2.16	2.13	2.12
4	2.19	2.16	2.20
5	2.18	2.17	2.16
6	2.20	2.18	2.19
7	2.23	2.21	2.18
8	2.25	2.24	2.27
9	2.27	2.30	2.29
10	2.33	2.31	2.30

The teeth in all groups were restored in the following way: The type 2 light-hardening powder and liquid glass ionomer cement (Variglass VLC, Dentsply, USA) were prepared in accordance with the manufacturer's instructions, applied to the bottom of the preparations and polymerized. (Astralis3, Vivadent, Australia). Following polymerization, all preparations were filled with a high copper amalgam (Cavex Avalloy, Cavex Co., Holland) and 24 hrs later finishing and polishing were performed.

The specimens were subjected to thermo cycling between 5ºC ± 4ºC and 60ºC ± 4ºC for 500 cycles. After thermo cycling, the surface of the teeth, up to approximately 1.5 mm to the restoration, was coated with a layer of nail varnish, melted utility wax and a second layer of nail varnish (Derhami et al. 1995; Iwami et al. 2000; Gungor et al. 2003; Yavuz et al. 2003; Olmez et al. 2004; Yavuz and Aydin, 2005).

The methylene blue (MB) solution was prepared to a concentration of MB 4.75 g/l. A stock solution was prepared using a buffer of $H_2PO_4^-$ / HPO_4^{-2} (phosphate / biphosphate) with a pH of 6.98 and 24 hrs did storage the specimens in the MB solution. Section made at the middle of the restorations to examine dye penetration.

In our study, the first stage was to evaluate the marginal leakage of specimens to confirm using the dye penetration test (microscope at a magnification of x 25). The results were evaluated using the microleakage score; 0 = no dye penetration; 1 = dye penetration between the restoration and the tooth up to one-third of distance between the tooth surface and the axial wall; 2 = dye penetration extending beyond one-third of the distance between the tooth surface and the axial wall; 3 = dye penetration extending two-thirds of the distance between the tooth surface and axial wall; 4 = dye penetration reaching the axial wall; and 5 = dye penetration reached the allof axial wall (Figure 2, Table 1) (Yavuz and Atakul, 2000; Yavuz and Atakul, 2001a; Yavuz and Aydin, 2005).

The second stage of this research was the measurement of the volume of the marginal gaps. In this stage, each individual sample was quantitatively measured using the chemical molecular characteristic properties of MB.

Theoretically, the volume measurement method was created and applied as described below.

The MB molecule is made up of an acid combined with an organic base. Its molecular weight is (M_A=319.868g.mol^{-1}) and a single piece of the absorbed covers an area of (s)=120 A^{0} ^{2} on the surface (Aydin and Tez, 1996) (Figure 3).

Absorption is the accumulation of dissolved molecules over the surface of a solid matter, the dissolved molecules could be atoms or ions of matter present in any solution of a gas, vapour or liquid phase. The phase, which allows the accumulation to occur on its surface, is known as the absorber (the teeth), the matter, which accumulates, is known as the absorbed (MB) (Davies, 1952; Nelsen et al. 1952; Aydin and Tez, 1996).

Absorption, in the liquid form, is usually measured using an indirect method. After the experiment, the teeth were dissolved in a 50% solution of nitric acid, the MB that filled the microleakage gaps dissolves into the solution and it's the MB concentration is determined.

To draw the calibration graph, a part of the MB solution was taken and determined to have a wavelength of 664 nm in a spectrophotometer l_{max} (maximum absorption wavelength).

Some of the MB stock was taken and diluted (10 different concentrations were prepared using 100 ml of distilled water in each beaker to dilute the 2% MB, ranging from 0 mL added to 180 mL added in 20 mL increments) to form a series of solutions of varying concentration. These varying concentrations were measured for their absorption wavelengths. These measurements were then used to construct the calibration graph (Figure 4): A = E C (A: absorption, E: molar absorption coefficient, C: concentration).

The molar absorption coefficient was determined to be $170.57 \text{ dm}^3.\text{cm}^{-1}.\text{g}^{-1}$.

After the calibration graph was drawn, the concentrations were calculated using the absorption values.

In the absorption experiments of our study, the teeth were placed in three joje balloons along with 100 ml of MB solution. The teeth were subjected to MB dye penetration at 37°C for a 24 hrs period, to allow dye penetration into any possible existing gaps between the tooth substance and the restorative material (Yavuz and Atakul 2000; Yavuz et al. 2003; Yavuz and Aydin, 2005).

Afterwards, the tooth tissue around the restoration surfaces was removed in a block by making cuts 1.5 mm around the restored area (Figure 5, Figure 6). These blocks were then dissolved separately in 50% nitric acid (Figure 7) and the amount of MB absorption was calculated using the previously created calibration graph. These values were converted to volume values (V = m/d) and the individual tooth volume values are reported in Table 2.

FINDINGS

Table 1 shows the scores of the degree of dye penetration for all groups. Measurement values of the MB staining in groups following the volume measurements of the three groups and the MB molecular counts equivalent to median values are shown in Table 2.

In our study, the first stage was dye penetration. We used Kruskal-Wallis non-parametric test. The differences were not statistically significant between all groups ($p > 0.05$).

In the second stage of this research, another three groups were evaluated statistically for microleakage volume at the restorations/cavity wall interface. The measurements in all groups were compared using One Way ANOVA Test and groups had no statistically significant differences ($p > 0.05$).

DISCUSSION

The *in vivo* microleakage phenomenon and the adaptation of filling materials into the cavity walls under clinical and laboratory conditions constituted the focal points of researchers for many years and a variety of methods have been used to research this (Tjan and Tan, 1991; Yavuz and Atakul, 2001b; Yavuz, 2003; Kelsey et al. 2004; Turgut et al. 2004; Ersin and Eronat, 2005). Some of these laboratory models have been successfully used to in order to determine microleakage, but they are not quantitative methods.

It is interesting that, despite the effect microleakage has on the health of dental pulp was established, little progress has been made in characterizing the dynamics and nature of microleakage.

In fact, in the studies of dye penetration, the dentin staining was observed to be more different than the actual gaps between cavity walls and restoration materials. This resulted in the use of a dye with a particle diameter equal to the bacterial size or smaller by researchers (around 2 μm) (Yavuz and Aydin, 2005).

In this study, a 2.00% solution of the MB molecule was used (one MB molecule = $1.2 \text{ nm}^2 = 120 \text{ A}^{0\ 2}$) since the particle size is less than that of the bacterial one. MB molecules were used because the also dissolve as monomer and bimer in an aqueous environment in which the pH is adjusted to 6.98 with a phosphate and biphosphate buffer (Nelsen et al. 1952; Yavuz and Aydin, 2005).

Another important issue in microleakage studies arises from the scoring systems. Since the evaluation in those studies largely depends on the observer's interpretation, the leakage scoring is at best a semi-measurable method (Yavuz and Aydin, 2005).

Various studies performed show that the dye leakages in different sections taken at different places of the restorations may show significant differences (Yavuz and Atakul, 2001b). For this reason, the accuracy of a leakage study based on a single section made from a tooth may be negligible.

As of today, there are no quantitative methods applicable and valuable for the microleakage determination; we have above indicated the amount of microleakage through quantification.

In the stereo microscopic studies, the method is based on the interpretation of the leakage of dye on the cavity wall and is defined as a semi-quantitative approach where the leakage is calculated solely at the surface where the section is made (Yavuz and Atakul, 2001b; Yavuz and Aydin, 2005).

In our method, the researcher's observation and interpretation do not come into play in the determination of microleakage volume quantity and all surfaces where a leakage occurs between tooth/restoration material is quantitatively measured by a chemist.

When the three groups were compared for microleakage volume measurements using the One Way Anowa Test, there weren't statistically significant difference ($p > 0.05$), also the dye penetration test (control groups), evaluated using the Kruskal-Wallis non-parametric test confirmed our method and the results had no significant difference ($p > 0.05$).

The comparison and/or relationship between the results obtained from the new method and the conventional method (control group) has been found similar.

The aim of this study was to develop an *in vitro* model to determine the microleakage volume. As a result of this study, the relative microleakage volumes of dog and bovine permanent teeth to human permanent teeth in *in vitro* conditions was found to be similar.

Within the limitations of these experiments, the following can be concluded, the ability to use dog and bovine teeth instead of human teeth in *in vitro* studies was confirmed.

It was also concluded in this preliminary investigation that the method of measuring the microleakage volume can be best a valuable tool for the *in vitro* study of microleakage dynamics around dental restorations, and this method can be use as a new technique for the determination of microleakage volume.

Further work to establish the true scope of the model remains to be undertaken, but this preliminary investigation shows promise.

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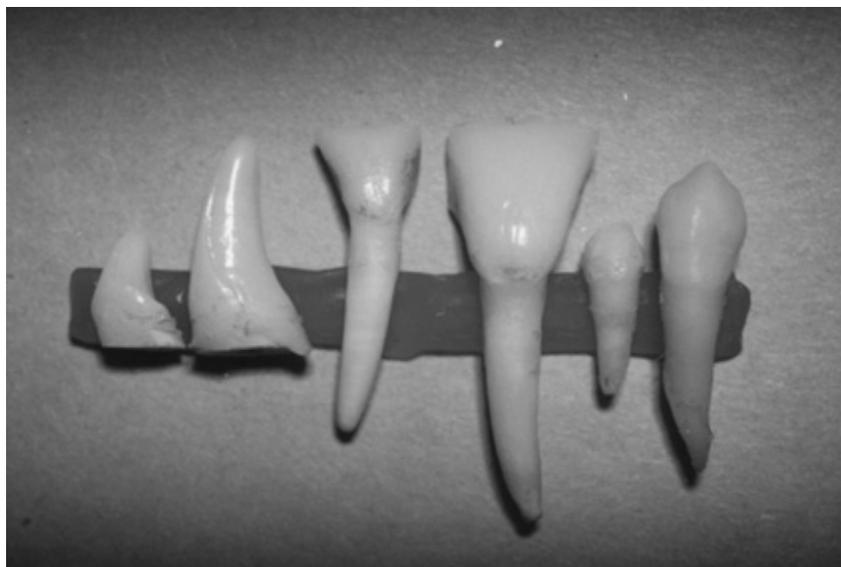
APPENDIX

FIGURES

Figure 1. Teeth of dog, bovine and human from left to right side respectively.

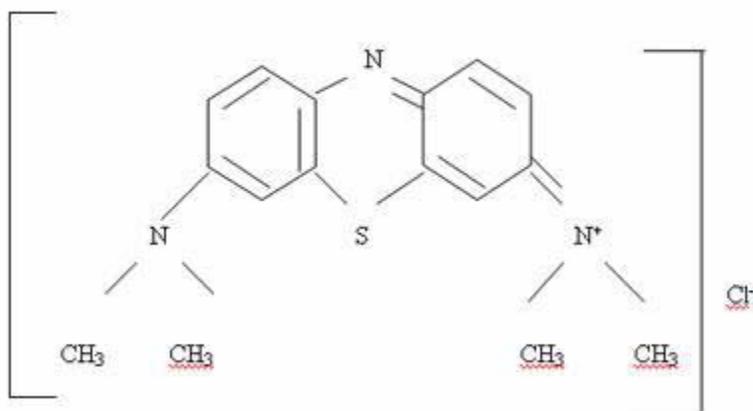**Figure 2. Dye penetration specimen (Magnification x 40).**

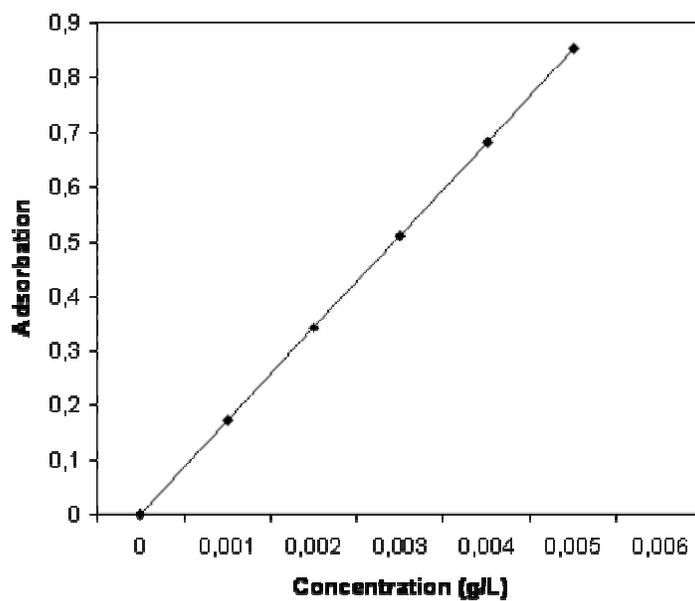**Figure 3. The molecular structure of methylene blue.**

Figure 4. The calibration curve of methylene blue.
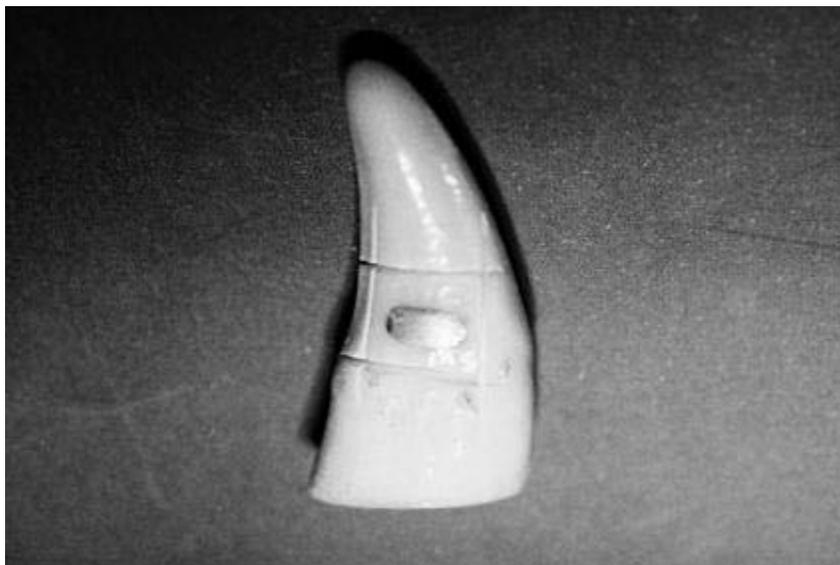
Figure 5. Appearance of cutting model of a specimen.

Figure 6. The block of a specimen obtained from tooth (Magnification x 10).

Figure 7. View of melted teeth block specimens in 50% Nitric acid solution.