Cytotoxicity and Dentin Permeability of Carbamide Peroxide and Hydrogen Peroxide Vital Bleaching Materials, *in vitro*

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There has been recent concern about the inadvertent exposure of dentin with patent tubules as well as gingiva to bleaching systems containing 10-15% carbamide peroxide or 2-10% hydrogen peroxide for more than a few minutes. The aims of the present study were: (1) to determine the cytotoxicity of dilutions of hydrogen peroxide in cell culture; (2) to measure hydrogen peroxide diffusion from bleaching agents through dentin *in vitro*; and (3) to determine the risk of hydrogen peroxide-induced cytotoxicity from exposure of dentin to these vital bleaching agents. The 50% inhibitory dose (ID_{50}) of hydrogen peroxide to succinyl dehydrogenase activity in cultured cells was found to be 0.58 mmol/L after 1 h. All bleaching materials demonstrated diffusion of hydrogen peroxide through dentin in an "*in vitro* pulp chamber" device. The one- and six-hour diffusates of all bleaching agents through 0.5-mm dentin exceeded the ID_{50} in monolayer cultures. Inhibition of succinyl dehydrogenase activity corresponded to the amount of hydrogen peroxide that can rapidly diffuse through dentin *in vitro* and reach concentrations which are toxic to cultured cells in less than 1 h.

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Introduction.

Various bleaching techniques have been used over the years to obtain subjectively whiter teeth. Early methods included chloride of lime and soda (Dwinelle, 1850), nitric acid (Fitch, 1861), and sulphurous acid (White, 1861). Hydrogen peroxide (H_2O_2) was introduced as an irrigant for disinfecting alveolar abscesses and necrotic teeth (Harlan, 1882). At that time, it was suggested that it also might be useful as a bleaching agent for discolored teeth.

The materials most commonly used for current in-office bleaching techniques are a concurrent application of 30% H_2O_2, sodium perborate, and heat, after rubber dam isolation, with methods differing little from those of Prinz (1924) and Ames (1937). More recently introduced techniques allow for patient application of bleaching agents outside the dental office. Most of the new techniques rely on a relatively long-term application, *e.g.*, several hours a day for several weeks, of a bleaching agent to the teeth in a mouth tray custom-fitted by the dentist, but applied by the patient. The active ingredients of these agents are commonly either a 10-15% carbamide peroxide or a 2-10% H_2O_2 solution in a viscous base. A 10% carbamide peroxide solution contains 3.62% H_2O_2 and 6.38% urea. Although generally positive results have been reported concerning the whitening ability of these agents, few investigators have addressed the possible pathophysiological effects on oral and pulpal tissues from long-term treatment. Claims of safety have been based largely on past use of these peroxides as a short-term mouthrinse adjunctive to routine oral hygiene procedures. The concern of the present study was the longer-term effects on pulp, when peroxides are applied to patent dentinal tubules. The dental pulp is vulnerable through exposed dentin in patients with pulpal hypersensitivity, gingival recession, attrition, cervical abrasion, and leaking restorations, and gingiva is exposed directly to gels which leak from the trays. The aims of the present study were to: (1) determine the cytotoxicity of H_2O_2 solutions applied directly to cell monolayer cultures, (2) measure H_2O_2 diffusion from bleaching agents through dentin, and (3) study the risk of H_2O_2-induced pulpal cytotoxicity because of exposure of dentin to these vital bleaching agents.

Materials and methods.

Three series of experiments were performed. The first measured the responses of cellular succinyl dehydrogenase to a series of concentrations of stock H_2O_2. The second utilized the bleaching agents in an "*in vitro* pulp chamber" (IVPC) device to determine the amount of H_2O_2 that diffused through 0.5 mm of dentin following application of the bleaching agent. The third measured the cellular response to the bleaching agents after diffusion through dentin.

Preparation and assay of hydrogen peroxide solutions.— So that a standard curve would be established, volumetric dilutions of H_2O_2 (30% w/v; Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS) were analyzed colorimetrically by means of the leucocrystal violet/horseradish peroxidase (LCV/HRP) assay (Mottola *et al.*, 1970). This method was used for determination of the concentration of H_2O_2 in tissue culture medium as well as diffusates through dentin. The LCV solution was prepared by dissolution of 30 mg of leucocrystal violet (Sigma) in 48 mL of 0.5% (v/v) HCl and dilution of that to 60 mL with acid of the same strength. Acetate buffer was made by mixing equal volumes of 2 mol/L sodium acetate and 2 mol/L acetic acid, and then adjusting to pH 4.5 with glacial acetic acid. HRP solution was made by dissolution of 10 mg of horseradish peroxidase (200 purpurogallin units/mg; Sigma) in 10 mL of distilled water. All solutions except the

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TABLE 1
BLEACHING MATERIALS

Product (Manufacturer)	*Active Bleaching Ingredient	**Concentration of Bleaching Ingredient
BriteSmile™ (BriteSmile, Birmingham, AL 35233)	HP	3% & 10%
Denta-Lite™ (Challenge, Osage Beach, MO 65065)	CP	10%
Dentlbright™ (Cura, Jacksonville, FL 32216)	CP	10%
Rembrandt Lighten™ (Den-Mat, Santa Maria, CA 93456)	CP	10%
Union Broach Nu-Smile™ (Union Broach, New York, NY 10036)	CP	15%

*HP = Hydrogen Peroxide; CP = Carbamide Peroxide.

**A 3% hydrogen peroxide solution contains 0.88 mol/L H_2O_2. A 10% carbamide peroxide solution contains 3.62% H_2O_2 (1.06 mol/L). Other concentrations are proportionate.

buffer were kept at 4°C during storage and were stable for at least 7 d. Aliquots of LCV solution (0.5 mL), HRP solution (0.25 mL), buffer (2 mL), H_2O_2 solution (0.5 mL of concentrations ranging from 0 to 0.88 mmol/L), and double-distilled water (1.75 mL) were added to make a total volume of 5.0 mL. The tubes were shaken and allowed to sit for 5 min, followed by 5 s of being vortexed. Light absorbance was measured at 596 nm on a Beckman DU-64 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) against PBS as a blank. Four experimental replicates were made for each dilution of H_2O_2 in PBS. PBS without H_2O_2 served as the negative control.

Cytotoxicity of hydrogen peroxide.—Balb/c 3T3 fibroblasts (clone A31; American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 3% NuSerum (Collaborative Research, Bedford, MA), supplemented with 1 μL/mL gentamycin (Flow Laboratories, Inc., McLean, VA), as well as supplemental penicillin-streptomycin and glutamine. These cells were passaged every third day until they were used for experiments. At the time of the assay, the fibroblasts were plated at 40,000 cells/cm² in 96-well cell-culture dishes (Costar, Cambridge, MA) in 100 μL of medium *per* well. After addition of the cells, the dishes were kept in an incubator with an atmosphere of 5% CO_2, 95% air, and 85% relative humidity at 37°C for 24 h prior to the experimental procedures.

Aliquots of H_2O_2 were diluted in supplemented DMEM to make concentrations ranging from 0 to 16 mmol/L. After removal of the old medium, new medium containing H_2O_2 was placed on the cultures, and they were returned to the incubator for 1 or 6 h before enzyme histochemistry was performed. The pH of the medium was not altered by the H_2O_2 addition and remained between 7.2 and 7.4. At the time of the succinyl dehydrogenase (SDH) assay, the medium was removed and immediately replaced with a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma; 100 μL/well) in the presence of disodium succinate as described previously (Wataha *et al.*, 1991). The formazan reaction product, when extracted with 6.25% 0.1 mol/L NaOH in dimethylsulfoxide, is stable for 1.5 h at 25°C. Eight experimental replicates were made for each dilution of H_2O_2. Simple least-squares linear regression was used for determination of whether there was a relationship between cytotoxicity of the diffusates of bleaching agents and the concentration of H_2O_2 present.

Diffusion of H_2O_2 through dentin.—To determine H_2O_2 diffusion through dentin, bleaching materials were applied to dentin within the IVPC illustrated in Fig. 1. The LCV/HRP assay was used for determination of the H_2O_2 concentration in the diffusate. The bleaching agents tested are listed in Table 1. The version of IVPC used in these experiments was a modification of the split-chamber device described by Outhwaite *et al.* (1974). In the IVPC, the dentin surface area for diffusion was 0.29 cm² as defined by the silicone O-ring. The lower chamber volume was 0.5 mL. Dentin disks, 0.5 mm thick, were cut

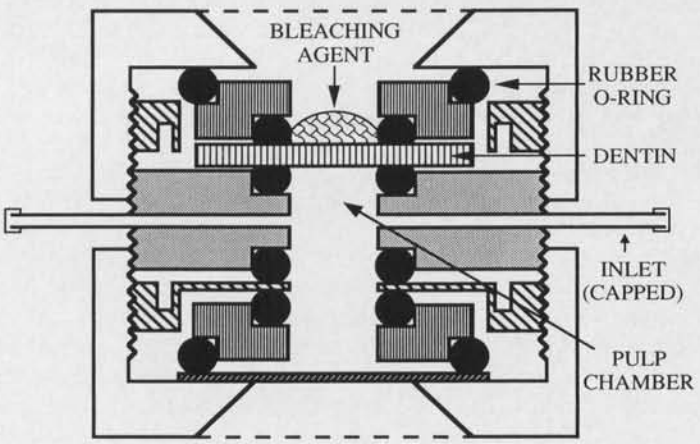

Fig. 1—Schematic of the "*in vitro* pulp chamber" (IVPC) used in this study. All parts were fabricated from polycarbonate except for the silicone rubber O-rings, the dentin disk, and the plastic cover slip in the bottom of the chamber. The lower chamber, representing the pulp chamber, was filled with 500 μL of PBS, which also saturated the tubules of the dentin disk. Approximately 100 μL of each bleaching agent was placed on the coronal surface of the dentin disk for the diffusion period.

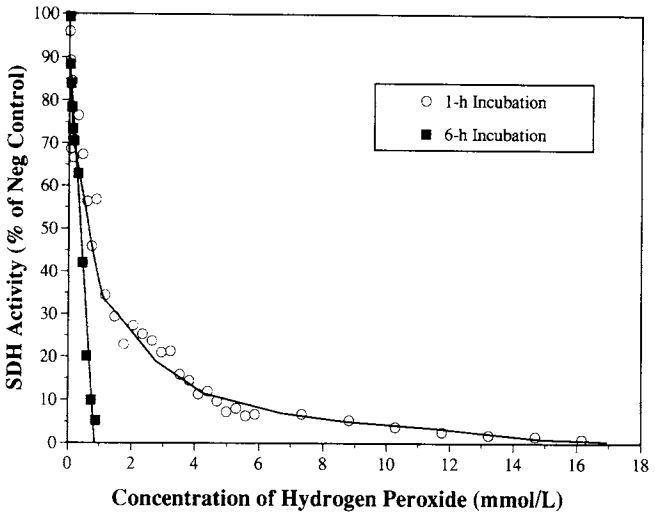

Fig. 2—Response of Balb/c 3T3 fibroblasts to serial dilutions of stock H_2O_2. The one- and six-hour incubation responses are in terms of SDH activity as a percent of negative control values.

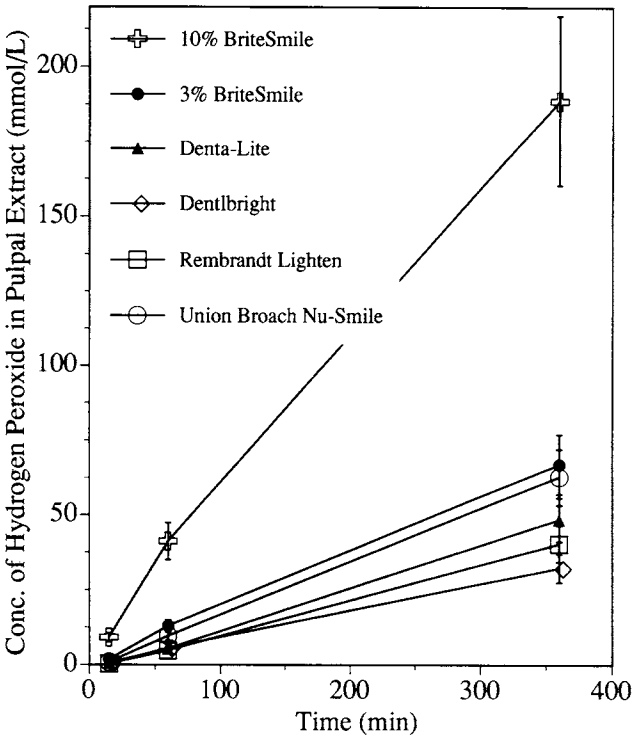

Fig. 3—Concentration of H_2O_2 within the lower chamber of IVPC #3 (one of six IVPCs used), after diffusion of bleaching materials through the dentin for 15 min, 1 h, and 6 h. These results are similar to those with all other IVPCs.

from extracted, non-carious permanent molar teeth of young human adults, smoothed with 600-grit sandpaper, treated for 2 min with 0.5 mol/L EDTA (pH 7.4), and stored in 70% ethanol until used, as described by Hanks *et al.* (1989). Dentin disks were initally stored in 70% ethanol to discourage bacterial growth and to stabilize biological membranes within the dentinal tubules with minimal volume change of anatomical structures (Hayat, 1970). The hydraulic conductance (Lp; μL/cm²/min/cm H_2O) for each disk was determined at room temperature (21°C) by use of a 180-cm manometer column filled with 18 MOhm of water. Six dentin disks were assembled in separate IVPCs, and the positions of the disks remained unaltered throughout all procedures. This resulted in more reproducible data among all procedures with any single IVPC by limiting the influence of regional variations in permability across a section of dentin (Pashley *et al.*, 1987). Before each experiment, the lower section of each IVPC was partially disassembled so that the pulp chamber could be filled without disturbing the position of the dentin. The IVPCs were then rinsed under 45°C tap water for 10 min, re-assembled, and run under the manometer for 15 min. The IVPCs were then filled with PBS and placed under house vacuum (0.1378 MPa; 1400 cm H_2O) for another 10 min to replace air bubbles in the dentinal tubules with PBS. Pilot studies with UV-VIS spectrophotometry revealed that these procedures were adequate for the removal of unbound residual ethyl alcohol or bleaching agent in the dentinal tubules prior to each experiment. After the bleaching agent was placed on the coronal surface of each dentin disk in its IVPC at room temperature, the IVPCs were incubated at 37°C for 15 min, 1, or 6 h. Afterward, the lower portion of the IVPC was again removed, and each diffusate was removed and placed in a borosilicate test tube, vortexed for 10 min, and then assayed for H_2O_2. Comparison of the optical densities for the diffusates with those for the H_2O_2 standard curve provided the H_2O_2 concentrations in the diffusates in mmol/L. The assays were always repeated.

Osmolarity of bleaching agents.—Osmolarity readings were made on 1/20 dilutions in 18-MOhm water of all bleaching agents by use of a model 31 LAS Osmometer (Advanced Instruments, Inc., Newton Highlands, MA). The instrument was calibrated with NaCl standards, and readings were extrapolated back to the original concentration of the bleaching agent as used clinically and expressed in units of mOsm/kg.

Cytotoxicity of dentin diffusates.—Cell monolayer cultures of Balb/c 3T3 fibroblasts were plated in 96-well dishes as described previously. The diffusates from the bleaching agents were obtained after 15 and 60 min, placed in borosilicate glass tubes, sealed, and refrigerated at 4°C. All diffusates from both time periods were assayed within 20 h. This allowed all bleaching materials from both time periods to be tested with the same passage of cells. Pilot studies established that the H_2O_2 content was stable within the medium for at least 3 d when stored in this manner. At the time of the assay, each borosilicate glass tube containing approximately 0.5 mL of diffusate was vortexed for 10 s. Aliquots (100 μL) of each diffusate were placed in each well containing a monolayer of cells for 1 h at 37°C. Then, succinyl dehydrogenase activity was assessed by the MTT assay. Negative controls were tested by the addition of PBS directly to the wells. An additional control involved the testing of dentin diffusates after application of 100 μL of PBS upon the dentin, instead of the bleaching agent. Enzymatic activity in the experimental wells was evaluated as a percentage of negative controls. Controls were treated in the same manner as the experimental groups to ensure that the IVPC assembly or dentin disks by themselves did not affect succinyl dehydrogenase activity.

Data from diffusion experiments and cytotoxicity experiments were compared by analysis of variance with

Tukey pair-wise comparisons at the 95% confidence level to determine similarities between agents as well as times. After the amounts of H_2O_2 diffusing through dentin had been determinined in at least two trials of each bleaching agent through six disks in IVPCs at each time interval (Fig. 3), coefficients of permeability (P; Outhwaite *et al.*, 1976) were determined for each time period from these same data as follows:

$$P = \frac{J}{C_1 - C_2}$$

where J = solute flux in mol of solution/min/cm which passes through dentin in a given period of time,

C_1 = conc. of solute in upper chamber in mol/cm^3,

C_2 = conc. of solute in lower chamber in mol/cm^3, and

P = permeability coefficient expressed as cm/min.

Results.

Hydrogen peroxide standard curve.—A second-order polynomial curve fit to the data points of the standard concentrations for optical density *vs.* H_2O_2. The assay was not usable above 0.44 mmol/L H_2O_2, because the optical density exceeded the sensitivity of the spectrophotometer.

Cytotoxicity of stock hydrogen peroxide.—Succinyl dehydrogenase activity of cultured Balb/c 3T3 cells was evaluated as percent of negative controls after application of serial dilutions of H_2O_2 to cell monolayer cultures (Fig. 2). Depression of succinyl dehydrogenase (SDH) activity increased with increasing H_2O_2 content as well as time. At 1 h, the ID_{50} value for H_2O_2 was approximately 0.58 mmol/L. For the six-hour incubation period, the enzymatic activity dropped much more precipitously (ID_{50} = 0.44 mmol/L), with only 5% of the activity remaining after exposure to 0.88 mmol/L H_2O_2.

Diffusion of H_2O_2 through dentin.—Hydrogen peroxide readily diffused through dentin. Fig. 3 illustrates the diffusion pattern of the bleaching agents using IVPC #3, one of six replicate IVPCs (Table 2). The concentration of H_2O_2 in the diffusates increased with time for all bleaching agents. BriteSmile™ (10%) produced the greatest concentration of hydrogen peroxide within the "pulp" chamber for all diffusion times, followed by 3% BriteSmile™ and 15% Union Broach Nu-Smile™, respectively. Concentrations of H_2O_2 from Denta-Lite™, Dentlbright™, and Rembrandt Lighten™ were not significantly different from each other at any time period. By 6 h, H_2O_2 diffusion from 10% Brite-Smile was significantly greater than that from other agents. Next highest were 3% Brite-Smile and U.B. Nu-Smile, which were significantly greater than the last three agents at this time period.

H_2O_2 diffusion through each of the six dentin disks was similar for each time period. Fig. 4 illustrates one material, Rembrandt Lighten, tested with the 6 different dentin disks at the 15-minute, one-hour, and six-hour diffusion times. Thicknesses of all 6 dentin disks were chosen to be relatively uniform, with a thickness of 0.515 mm ± 0.018 mm (mean ± S.D.). The Lp values measured with the manometer ranged from 0.0667 to 0.1009 μL/cm^2/min/cm H_2O prior to all experiments (Table 2). Therefore, the range of Lp values for the dentin disks prior to experiments varied only by a factor of 1.5. When measured after experimental procedures, Lp values of the individual dentin disks had not changed by more than 15%, suggesting that convective permeability was not altered by H_2O_2 or the bleaching agents. Thus, comparisons of data could be made between multiple experimental runs for any single IVPC.

Cytotoxicity of dentin diffusates.—The average re-

TABLE 2

THICKNESS AND MEAN Lp* READINGS OF DENTIN DISKS

Pulp Chamber #	Dentin Disk Thickness (mm)	Lp Before Experiments†	Lp After Experiments†	% Change in Lp
1	0.52	0.089	0.086	-2.7
		(0.006)	(0.010)	
2	0.50	0.068	0.073	+10.0
		(0.012)	(0.0003)	
3	0.53	0.081	0.076	-6.8
		(0.012)	(0.006)	
4	0.50	0.085	0.097	+15.1
		(0.008)	(0.017)	
5	0.50	0.101	0.110	+8.8
		(0.006)	(0.010)	
6	0.54	0.077	0.082	+7.3
		(0.006)	(0.006)	

*Lp = conductance of water through the disk with units = μL/cm^2/min/cm H_2O.

†Expressed as Mean (S.D.).

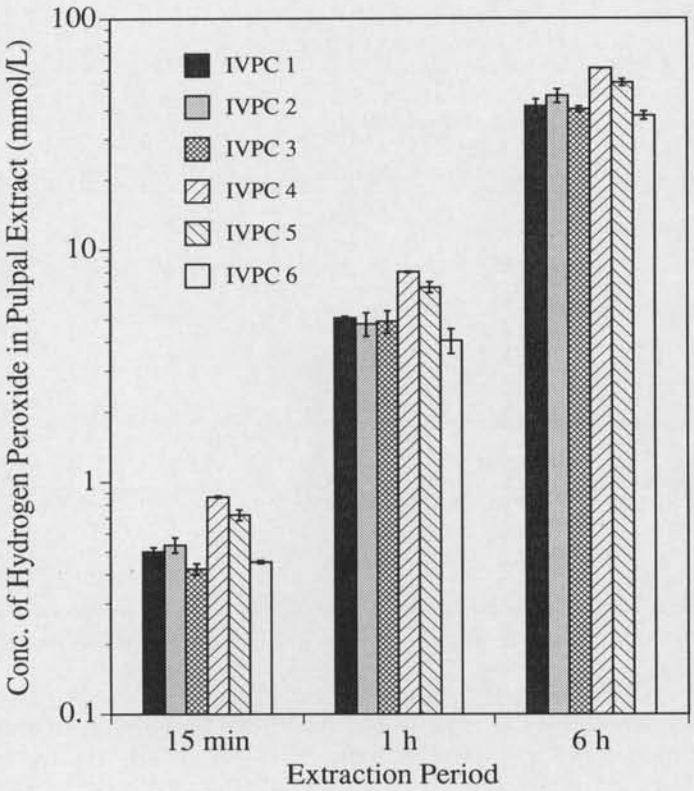

Fig. 4—Concentrations of H_2O_2 in the lower chamber of each IVPC after 15 min, 1 h, and 6 h of diffusion of Rembrandt Lighten through the dentin disks. The values represent the mean of two determinations (± S.D.).

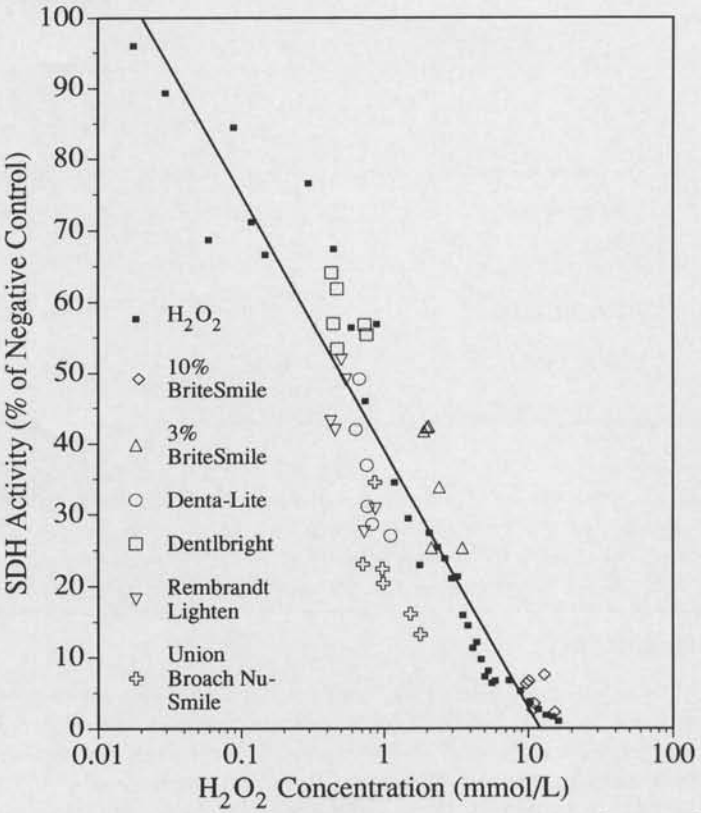

Fig. 5—Biological response (average of the percent of succinyl dehydrogenase activity in negative controls) of Balb/c 3T3 fibroblasts to 15-minute diffusates of six bleaching agents in comparison with the effects of serial concentrations of H_2O_2. Each point represents the mean of four determinations for each bleaching agent within a dentin-IVPC assembly, or of eight determinations for the serial concentrations of H_2O_2.

sponses of Balb/c 3T3 fibroblasts to 15-minute diffusates (*i.e.*, that part of the mixture of chemicals in the bleaching agents which have diffused across the dentin within 15 min) of the various bleaching agents showed substantial depression of SDH activity by all agents (Fig. 5). Only diffusates of 10% BriteSmile caused SDH activity to drop to less than 10% of controls. SDH activity of the 15-minute diffusates of the other agents was between 10 and 64% of negative control values. The coefficient of determination (r^2) for this line was 0.94, indicating that, for the total number of 15-minute diffusates from all IVPCs, there was a strong relationship between H_2O_2 concentration and cytotoxicity to Balb/c 3T3 cells. For one-hour diffusates, the average SDH activities of these fibroblasts were at or below 10% of negative controls for all bleaching agents from all 6 IVPC-dentin disk assemblies. Since six-hour diffusates contained even more H_2O_2, SDH activity after 6 h would also be at or below 10% of the negative controls. Thus, cytotoxicity tests for six-hour diffusates (cytotoxicity > 90%) were not performed.

Permeability coefficients, osmolarity, and pH of bleaching agents.—Several factors were studied to determine whether they correlated with the cytotoxicity which was measured in the cell test system as a depression of succinyl dehydrogenase activity. Of all the parameters tested, the concentration of H_2O_2 in the original bleaching agent correlated best ($r^2 = 0.702$; Table 3). Other factors tested were permeability of the dentin to H_2O_2, osmolarity of the bleaching agents, and pH of the bleaching agents. Permeability coefficients (*P* values) for 15-minute diffusates for each agent are shown in Table 3. The 10% BriteSmile gave the largest *P* value, followed by 3% Brite Smile and then Denta-Lite and U.B. Nu-Smile. These permeability coefficients for 15-minute H_2O_2 diffusates from the bleaching agents did not correlate very well with cytotoxicity ($r^2 = 0.503$). For 60-minute diffusates, the correlation between SDH activity (below 5% of negative controls) and the permeability coefficients (data not shown) was less ($r^2 = 0.327$).

Another factor tested for correlation with cytotoxicity was the osmolarity of the bleaching agents (Table 3). Denta-Lite had the highest value, followed by Rembrandt Lighten. As controls for the osmolarity readings of the bleaching agents, values for other materials—including human saliva, PBS, F-12 tissue culture medium without serum, and 18-MOhm water—were tested and found to be 92, 295, 528, and <20 mOsm/kg, respectively. The correlation between osmolarity of the agents and cytotoxicity was not as good ($r^2 = 0.36$) as that for permeability of the 15-minute diffusates.

A third factor was the pH of the bleaching agents. While several of the bleaching agents were either alkaline or very acidic before diffusion, the pulpal extracts were all well buffered by the tissue culture medium and within a pH range which would not cause cytotoxicity. The pH values of the diffusates are shown in Table 3 for 15-minute and 60-minute diffusates. The pH value of the tissue culture medium was maintained in a range close to pH 7.2-7.6.

TABLE 3

TOXICITY *vs.* OSMOLARITY, PERMEABILITY, pH, AND H_2O_2 CONCENTRATIONS

Bleaching Agent	*SDH % of Neg. Cont. Mean (S.D.)	**P Perm. Coeff. (cm/min)	Osmolarity (mOsm/kg)	pH of Bleaching Agents	***pH Diffusates
10% BriteSmile	4.8 (2.3)	0.00043	6700	7.39	7.45-7.57
3% BriteSmile	35.1 (8.2)	0.00030	4900	8.25	7.48-7.55
Denta-Lite	35.7 (8.6)	0.00010	55000	3.30-3.42	7.33-7.56
Dentlbright	58.0 (4.0)	0.00007	11400	5.04-5.30	7.23-7.41
Rembrandt Lighten	40.6 (9.7)	0.00007	25000	5.50-6.25	7.24-7.39
U.B. Nu-Smile	21.5 (7.4)	0.00010	14500	5.70-5.80	7.13-7.36

*Cytotoxicity of 15-minute diffusates of bleaching agents after passage through dentin in terms of succinyl dehydrogenase activity in cell assay system.

**P = permeability coefficient.

***Range of pH values for 15- and 60-minute diffusates.

Discussion.

The inhibitory effects of standard hydrogen peroxide solutions on the succinyl dehydrogenase activity of Balb/c 3T3 cells were analyzed after one- and six-hour incubation periods (Fig. 2). The results indicated that cytotoxicity of H_2O_2 was influenced by both the concentration of the peroxide and the length of time it was in contact with the cells. Although the ID_{50}s for one- and six-hour incubations of H_2O_2 with Balb/c 3T3 cells were 0.58 mmol/L and < 0.44 mmol/L, respectively, these estimates of biological cytotoxicity may differ from those of other studies because of slightly different sensitivities of different cell types and different incubation periods. For example, Peterkofsky and Prather (1977) reported an ID_{50} of approximately 0.008 mmol/L H_2O_2 for chick embryo fibroblasts, but this was for 24 h of incubation with H_2O_2. Tse *et al.* (1991) applied either a 2 mg/mL Rembrandt Lighten with 10% CP or 3 mg/mL of the same agent with 15% CP (21 and 32 mmol/L H_2O_2, respectively) directly to cultured human umbilical-cord-vein endothelial cells and found that both H_2O_2 concentrations reduced MTT activity to approximately one-quarter (ID_{75}) of negative control level in 30 min. In the present experiments, a one-hour incubation of H_2O_2 with Balb/c3T3 cells gave an ID_{50} value of approximately 0.6 mmol/L H_2O_2 and an ID_{75} value of approximately 2 mmol/L (Fig. 2). These data are consistent with the findings of the other two previously cited studies.

Significant amounts of hydrogen peroxide diffused through dentin after application of carbamide peroxide- and hydrogen peroxide-based bleaching agents to the coronal surfaces of dentin disks placed in IVPC assemblies. The amount of hydrogen peroxide that diffused through the dentin was most dependent on its original concentration within the bleaching agent and the length of time the agent came into contact with the dentin (Fig. 3). The cytotoxicity to the cell test system resulting from H_2O_2 diffusion correlated fairly well with the original H_2O_2 concentration (r^2 = 0.70), but less well with the coefficient of permeability (r^2 = 0.50). Thus, only about 50% of the variation of the cytotoxicity caused by the bleaching agents can be explained by the permeability of H_2O_2.

It took as little as 15 min for H_2O_2 from the various bleaching agents to diffuse through 0.5 mm of dentin and reach a level capable of causing harmful biological effects. The P value for 10% BriteSmile (with 2.9 mol/L H_2O_2) was 4.3 x 10^{-4} cm/min (Table 1). This value is similar in magnitude to the value given by Outhwaite *et al.* (1976) for ^{125}I diffusion through 0.5-mm human molar dentin disks cut close to the pulp, where the diffusional surface area was 0.29 cm^2. As shown by Outhwaite *et al.* (1976), diffusional flux of a solute like ^{125}I or H_2O_2 is directly proportional to the surface area of exposed dentin (or tooth surface) and inversely proportional to dentin thickness. In the present study, the fact that the permeability coefficients for all H_2O_2-containing bleaching agents were not of the same magnitude indicates that the diffusion of H_2O_2 is complex.

At least two forces might be working against the diffusive flux of molecules from the bleaching agents toward the pulp: convection due to positive pulpal pressure and osmotic pressure of the gels. Matthews and Pashley (1992) showed that pulpal fluid pressure is capable of reducing inward diffusion of ^{125}I dramatically in the presence and absence of a smear layer. Although we did not determine the effect of either convection or osmotic pressure on H_2O_2 diffusion, we did measure the osmolarities of the various gels (Table 3). The bleaching agents were unaltered from the way they would be used clinically. Bleaching agents with higher osmolarity might not allow diffusion at the same rate as those with lower osmolarity, since the higher osmolarity would tend to draw fluid away from the pulp. However, Fig. 3 shows that there was very little difference in the diffusive flux of H_2O_2 from any bleaching agent except 10% BriteSmile. Although the H_2O_2 content of the other agents varied from 3% (BriteSmile) to 5.43% (Union Broach Nu-Smile), the osmolarity appeared to be controlled by the gel composition rather than by the peroxide concentration. Osmolarities of the bleaching agents ranged between 4900 (3% BriteSmile) and 55,000 mOsm/kg (Denta-Lite). The effective osmotic pressures of small molecules are extremely

small (about 10^{-4} of the theoretical osmotic pressures) for dentin (Pashley and Whitford, 1980). Thus, osmolarity did not appear to have much of an effect in reversing the diffusion of H_2O_2.

The diffusion data for H_2O_2 (Fig. 3, Table 3) suggest that, at the prescribed periods of 15 min and beyond, all of these bleaching agents were capable of diffusing through 0.5 mm of patent dentin and damaging pulpal tissue. These permeability data from 0.5 mm of dentin would apply mainly to areas of cervical erosion or abrasion, areas which also develop sclerotic dentin. However, if the teeth are "hypersensitive", this would indicate that the dentin tubules are patent, and this treatment would be contra-indicated on the basis of simple diffusion data. As used clinically, H_2O_2 is usually applied to intact teeth (often anterior) with and without heat treatment to accelerate the action. The thickness of dentin used in the present study is in the appropriate range for human anterior teeth, and thus cytotoxicity may pose a clinical concern. Variables to be considered before clinical application should include (1) whether dentin with patent tubules is exposed, (2) thickness and area of exposed dentin, (3) location of the exposed dentin, (4) inflammatory and microcirculatory status of the pulpal tissue, and (5) use of anesthetics. In addition, H_2O_2 may complicate the situation by causing hemostasis and vascular necrosis. In normal tissue, responses elicited by inflammatory mediators would usually result in increased blood flow and tissue pressure, facilitating removal of the inflammatory mediators and irritants. Pulpal tissue, with limited volume to expand, may have a compromised response to inflammatory stimuli because of tissue pressure associated with edema (Heyeraas and Kvinnsland, 1992). However, not all inflammatory mediators cause reduction of arterial blood flow in inflamed pulpal tissue, so that it is not clear whether dentin diffusates remain stagnant or are removed more quickly from the inflamed pulp (Heyeraas and Kvinnsland, 1992; Kim *et al.*, 1992).

There have been few reports of pulpal damage of intact teeth in humans and experimental animals exposed to H_2O_2 and bleaching agents. Cohen (1979) reported that treatment of 51 human premolars with 35% H_2O_2 (54°C) had little or no clinical effect, and there was no subsequent histological evidence of pulpal damage. Robertson and Melfi (1980) reported that a 35% solution of H_2O_2 for five-minute periods for 2 appointments, with heat ranging from 46-51°C, caused mild inflammation of a significant number of human pulps. Seale *et al.* (1981) treated canine teeth of young mongrel dogs with 35% H_2O_2 for 30-minute periods for each of 4 weekly periods, with and without heat treatment (62°C). While heat alone appeared to cause reversible blood vessel dilatation, H_2O_2 treatment alone was associated with complete obliteration of the odontoblastic layer, loss of predentin, a dense inflammatory infiltrate, areas of internal resorption, and pulpal hemorrhage within 3 days after treatment. By 60 days, the inflammatory changes were beginning to resolve, the internal resorption was being repaired, and the odontoblastic layer appeared to be recovering and assuming a columnar shape. The increased severity of reaction in young dog canine teeth as compared with those in the human studies was thought to be due to thinner dentin in the dog teeth.

Pathological vascular phenomena such as reduced pulpal blood flow, necrosis, and hemorrhage frequently seem to be caused by peroxides. Edwall and Olgart (1972) and Edwall (1974) described potentiated reduction of pulpal microcirculation with serial applications of 3% H_2O_2 to deep dentin cavities of cat canine teeth. While normally one would expect diffusates to be diluted by outward flow of interstitial fluid from the pulp due to intrapulpal tissue pressure, application of H_2O_2 may arrest this "washing" effect. Pohto and Scheinen (1959) reported that H_2O_2, when placed on deep dentin of rat incisors, caused gas emboli in the blood vessels of the pulp, and these vessels eventually ruptured. However, it is not known whether this same effect of H_2O_2 occurs with thicker dentin. With higher concentrations, vascular necrosis and hemorrhage may occur, as in the study by Seale *et al.* (1981). Stanley *et al.* (1979) reported similar pulpal hemorrhage following application of benzoyl peroxide to dentin tubules of monkey teeth, where the remaining dentin thickness was 0.95 mm.

Tse *et al.* (1991) argued that there are sufficient mechanisms which protect tissue from hydroxyl radicals generated from the reaction of H_2O_2 so that injury is usually not observed. Regardless of the mechanisms, there have been sufficient reports in the literature of diffusion of H_2O_2 through intact enamel (Atkinson, 1947; Wainwright and Lemoine, 1950; Arwill *et al.*, 1969; Bowles and Ugwuneri, 1987), and subsequent pulpal damage (Seale *et al.*, 1981; Seale and Wilson, 1985) to make the concern for application of bleaching to patent dentinal tubules reasonable. It is our view that tissue culture studies are important for understanding the unqualified dose-response phenomena in a toxicological sense, but that they may be limited in their ability to simulate the clinical conditions. Translation between clinical and laboratory permeability studies would be facilitated if both groups of investigators would apply the materials to a stated surface area and if dentin thickness was recorded. The present data demonstrate the potential for irritation after diffusion through 0.5-mm dentin with a surface area of 0.29 cm^2.

Finally, what implications does this study have for gingiva? One might expect the potential for damage to gingiva *in vivo* by these bleaching agents to be even greater because of direct contact of the bleaching agent with epithelium and connective tissues. For example, Rees and Orth (1986) have reported clinical ulceration caused by bleaching agents. The gingival response may have a latent period before it becomes apparent; Jonas *et al.* (1989) described a delayed response to H_2O_2 in epithelial cell cultures (CNCM I-221). However, in the presence of oral fluids, extensive gingival microcirculation, a high compliance environment, and other protective mechanisms, gingival tissues appear to be much less vulnerable than would be expected from the *in vitro* data. In fact, a concentration of 10% carbamide peroxide has been used as an adjunct for plaque control and for improving oral hygiene in orthodontic and periodontic patients (Shapiro *et al.*, 1973; Tartakow *et al.*, 1978). Clinical use of this adjunct treatment, however, has been for much shorter application periods (minutes), whereas bleaching techniques recommend several hours of application. The results of the present study suggest that longer (one-hour) clinical contact with gingival tissues should be more closely evaluated.

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