Time, concentration, and pH parameters for the use of glutaraldehyde as a pulpotomy agent: an in vitro study

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Abstract

The effects of time, concentration, and pH on glutaraldehyde (GA) fixation were studied in vitro using 2 model systems: collagen-BSA gels as simulations of cell cytoplasm and enzyme activity in treated pulp tissue. The former system demonstrated that GA is most effective when buffered, and that its penetration is self-limiting. In general, concentration proved more a factor in the depth of penetration than did time.

It was concluded from these experiments that buffering GA, increasing its concentration, and applying it for longer periods, all enhance the degree of fixation; only stronger solutions increase the depth of fixation. Practically, the data suggest that clinical treatment might involve using buffered glutaraldehyde — either at 4% for 4 min or 8% for 2 min.

Glutaraldehyde has received attention in recent years as an alternative to formocresol for pulp treatment of primary teeth (Ranly 1982; Kennedy 1986). It has demonstrated the following properties: superior fixation with relatively little immunogenicity (Ranly and Lazzari 1983; Ranly et al. 1985), mild effects on pulp tissue,[1] lesser systemic distribution (Myers et al. 1986), and positive clinical results (Garcia-Godoy 1986; Fuks et al. 1986).

The use of formocresol has evolved from multiple-appointment regimens using Buckley's full-strength preparation (Sweet 1930) to the current 5-min protocol with diluted formocresol (Morawa et al. 1975). The latter change, dilution of formocresol with glycerol and water, has been researched.[2] The other variable, length of application, also has received some attention recently (Garcia-Godoy et al. 1982).

To the authors' knowledge, glutaraldehyde has not been examined in the same way. In order to avoid empiricism in the use of glutaraldehyde, this study was undertaken to examine several variables which could influence its effectiveness as a pulpotomy agent. Accordingly, effects of pH, time, and concentration on glutaral-

[1] Kopel et al. 1980; Davis et al. 1982; Tagger et al. 1986.
[2] Straffon and Han 1970; Loos and Han 1971; Garcia-Godoy 1981.

dehyde fixation by several in vitro assays were investigated.

Methods

Preparation and Use of Collagen-BSA Gels

In order to have available a uniform and reproducible assay for the determination of the effects of pH, time, and concentration, a modification of the procedure developed by Flitney (1966) was used. Collagen gels (.3 g/2.8 ml) were prepared containing a physiologic salt solution and bovine serum albumin ([BSA] 1 g/10 ml). The mixture was dissolved in a boiling water bath and measured amounts were pipetted into plastic vials. For the first assay, gel wafers were removed from the vials and treated with control or glutaraldehyde solutions. In the second assay, entire vials with gels intact were submerged in the test solutions. Following treatment, the gels in the second assay also were removed from the vials. The relative fixation of the protein in the gels then was ascertained by measuring the amount of BSA which diffused from the gels into 4 ml of water during a 30-min period. BSA was quantified by the method of Bradford (1976).

Assay for Parameters Affecting Fixation

To determine the effect of pH, time, and concentration on the degree of fixation, collagen-BSA wafers were incubated in buffered and nonbuffered solutions of 0.05, 0.1, 0.2, 0.4, or 0.8% glutaraldehyde for 0.5, 1, 2, 4, or 8 min. Test solutions were prepared from 25% EM-grade glutaraldehyde; phosphate buffered solutions were adjusted to pH 7.1. Wafers treated with distilled water or buffer for identical time periods were used as respective controls. The quantity of BSA which diffused into the second solutions was determined and reported as percentage of control lost or retained. The mean and standard deviation were calculated for 5 samples in each group. Statistical differences between the groups were determined by Student's t-test.

Assay for Parameters Affecting Penetration

To determine the effect of time and concentration on the depth of penetration of buffered glutaraldehyde into collagen-BSA, vials containing gel were immersed in 0.5, 1.0, 2.0, 4.0, or 8% solutions for 0.5, 1, 2, 4, or 8 min. Preparation of solutions, handling of controls, sample size, and statistics were the same as in the previous assay.

Assay for Residual Enzyme Activity of Treated Bovine Pulp

Calf pulp was harvested from freshly extracted molars, minced into 1-mm³ pieces, divided into aliquots weighing approximately 0.5 g, and stored at -20° C until used. After thawing, each aliquot was wet weighed and incubated in 0.5, 1.0, 2.0, 4.0, or 8.0% solutions of buffered glutaraldehyde (pH 7.1) for intervals of 0.5, 1, 2, 4, or 8 min.

For the assay of residual lactate dehydrogenase (LDH) activity, the treated samples were homogenized for 1 min in 10 ml of 0.2 M Tris, pH 7.3, using a homogenizer. Following centrifugation for 10 min at 300 rpm on a clinical centrifuge, the supernatant was decanted. One hundred μl of the supernate was added to a cuvette containing 2.8 ml of Tris HCl, 1.0 ml of 6.6 mM NaOH, and 0.1 ml of 30 mM sodium pyruvate and vortexed for 10 sec. Change in absorbance per minute was recorded on a spectrophotometer at 345 nm at room temperature. The activities were calculated as units/mg of wet weight, and the mean and standard deviation were calculated for 5 samples in each group. All values are reported as a percentage of controls.

Results

Parameters Affecting Fixation

The influence of pH on the chemical reaction of glutaraldehyde is shown clearly in Figure 1. At the most

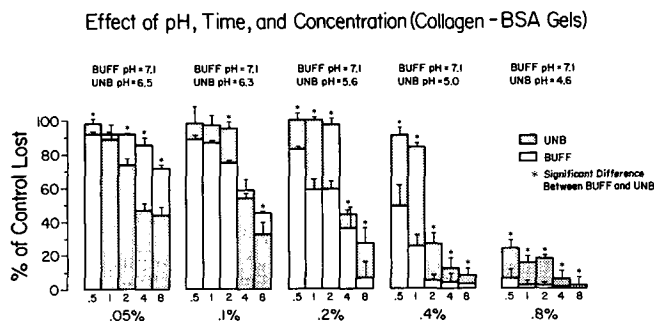Effect of pH, Time, and Concentration (Collagen - BSA Gels)

Fig 1. Effect of pH, concentration, and length of application of glutaraldehyde on the fixation of collagen-BSA gels.

dilute concentrations (0.05 and 0.1%), when the respective pH of the unbuffered glutaraldehyde solutions was 6.9 and 6.5, respectively, buffering did not improve fixation. At higher concentrations, however, when the un-

buffered preparations became more acidic, their ability to bind BSA and collagen diminished relative to buffered solutions. The graphs also display how the quality of fixation was enhanced in two ways — either by increasing the concentration or by lengthening the treatment at any one concentration. The results also demonstrate that concentration and time are additive with respect to the quality of fixation. After 8 min in the 0.8% solution, virtually no BSA is able to diffuse through the fixed surface of the gels.

Parameters Affecting Penetration

The results depicted in Figure 2 demonstrate that the depth of penetration of glutaraldehyde into a gel is primarily a function of concentration. At any given

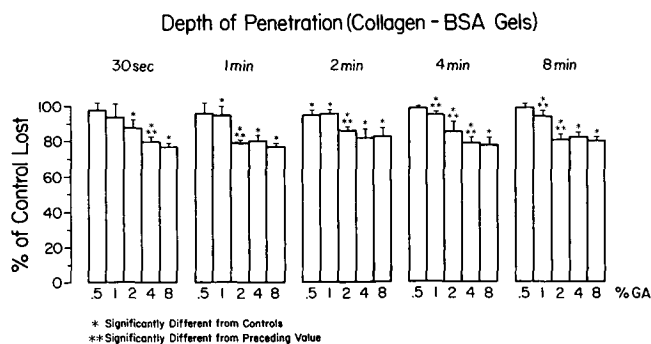Depth of Penetration (Collagen - BSA Gels)

Fig 2. Effect of concentration and length of application of buffered glutaraldehyde on the depth of fixation using collagen-BSA gels.

dilution, the length of treatment made no statistically significant difference in the amount of BSA bound by the advancing glutaraldehyde. The data also show that the penetration of glutaraldehyde was rather self-limiting; the maximum reduction in loss was only 20%, even by the highest concentrations. The zones of fixation of several preparations of glutaraldehyde can be compared visually in Figure 3, confirming the legitimacy of the biochemical assay for depth of penetration.

LDH Studies

The effect of glutaraldehyde on the residual activity of LDH in bovine pulp is summarized in Figure 4. The graphs clearly show that enzyme inhibition was increased by two factors — either higher concentrations or longer treatment periods at a given concentration. These two variables were additive; e.g., incubation of tissue in 8% glutaraldehyde for 8 min had the most profound impact on LDH activity. When the data are plotted as percentage of residual activity vs. log of concentration at each treatment period, the effect of glutaraldehyde is seen to follow log dose responses (Fig 5). Regression analyses of these plots resulted in values > 0.9 for all but the 8-min treatment period.
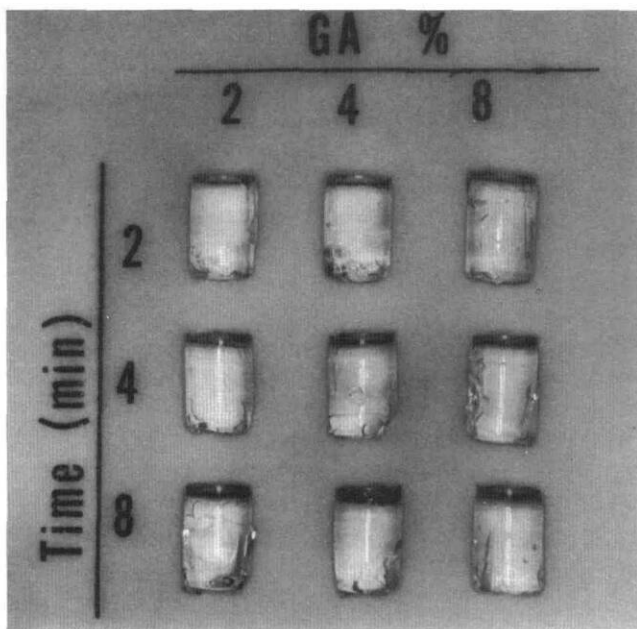
FIG 3. Collagen-BSA gels following application of glutaraldehyde at higher concentrations and time periods.

Effect of Buffered GA on LDH Activity

* Significantly Different from Controls
** Significantly Different from Preceding Values

FIG 4. Effect of concentration and length of application of buffered glutaraldehyde on LDH activity of bovine pulp.

Effect of Increasing Concentrations at Five Treatment Periods

FIG 5. Graphs of residual LDH activity for 5 constant time periods plotted against the logs of the concentrations. Regression analysis of the plots gave the following r values: 30 sec = 0.99; 1 min = 0.97; 2 min = 0.94; 4 min = 0.94; and 8 min = 0.87.

[3] Straffon and Han 1970; Loos and Han 1971; Garcia-Godoy 1982.

Discussion

Dentistry is replete with empirical application of compounds and drugs, one of the most notable examples being formocresol. Through the years, the time of contact of pulp tissue with formocresol has dropped dramatically, and currently a 5-min application is standard protocol. This reduction was apparently based on clinical judgment, without benefit of supporting biologic data. Only relatively late in the lifetime of formocresol have the effects of variables such as concentration and duration of application been studied biologically.[3]

Most dental studies of glutaraldehyde, both laboratory and clinical, have utilized 5-min treatment periods. This time period has no proven superiority over any other, and is obviously borrowed from the formocresol protocol. In addition, the most commonly used concentration, 2%, apparently was adopted from the electron microscopist. Thus, despite a growing awareness of the limited evaluation of the clinical variables of formocresol, history seems to be repeating itself with glutaraldehyde. Although this fixative is a relatively new agent being proposed as an alternative to formocresol, empiricism has already crept into laboratory and clinical investigations designed to evaluate it. Therefore, the authors decided to investigate systematically some of the parameters that could influence the clinical protocol for glutaraldehyde.

The authors' experience with the evaluation of pulp agents using pulp tissue has revealed the unavoidable variations in samples harvested from calves at different time periods. While a single experiment with appropriate controls can be completed satisfactorily with the same pooled tissue, the values cannot be compared directly to those obtained from different tissue harvested at another time. This deficiency complicates the comparison of certain kinds of data obtained from separate experiments; and it becomes particularly critical when the same preparation is being tested at several intervals, i.e., in an evaluation of shelf life.

Therefore, based on an in vitro system used by Flitney (1966) to compare a number of fixatives, the authors have developed what they consider to be a uniform and reproducible assay for glutaraldehyde cross linking. The collagen-BSA gel was formulated by Flitney to simulate the cytoplasm of the cell; BSA diffusion from the gel was intended to represent leakage of protein through the cell membrane during fixation. For the present study this methodology was adapted to analyze the effects of pH, time, and concentration on the extent and depth of fixation by glutaraldehyde.

The first experiment with dilute preparations clearly showed the enhancement of fixation by a neutral environment. The pH of unbuffered glutaraldehyde of medium concentration is normally in the 3.0-4.0 range, presumably as a result of the oxidation of aldehyde
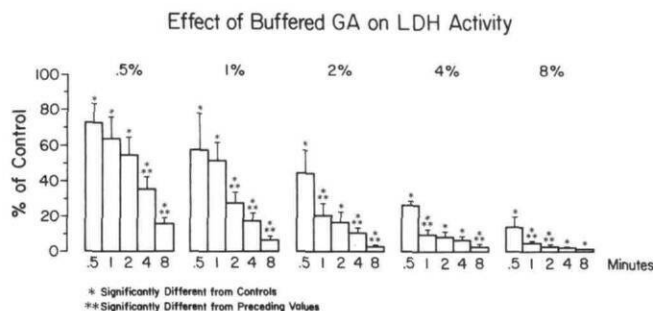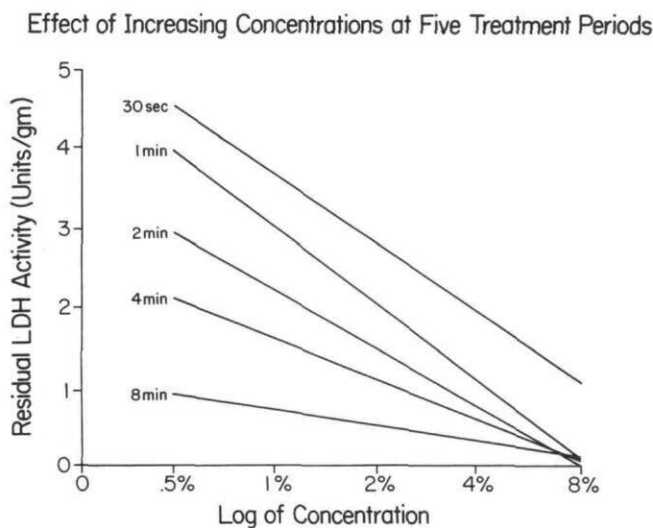
moieties to carboxyl groups. Some of the unbuffered solutions used in this study were so dilute that they did not undergo significant acidic changes; consequently, they exhibited equal or, in some cases, even better fixation than their buffered counterparts. However, when the unbuffered solutions reached decidedly acidic ranges, their ability to cross link protein was diminished significantly compared to buffered preparations.

This phase of the study also demonstrated that the extent or quality of fixation (based on the restriction of BSA diffusion from the gels) was promoted by either increasing the concentration of glutaraldehyde or increasing the length of application of any given preparation. Therefore, if in vivo testing verifies these findings, the protocol for glutaraldehyde can be manipulated significantly.

For clinical efficiency, application of higher concentrations of glutaraldehyde for short periods probably would be preferable to more dilute solutions for longer intervals. However, a recommendation to use higher concentrations might be tempered by future studies comparing the systemic distribution under different protocols.

Since it is the authors' contention that a localized, limited zone of fixation of pulp tissue in the vital pulpotomy is an ideal treatment objective, they were interested in the effect of time and concentration on the depth of penetration. For this phase of the study the gels were left in their vials so that only one surface was exposed to the incubation solution. Following treatment, the gels were removed and placed into water in order to monitor diffusion of BSA from the unfixed portions. In this assay, the depth of penetration should be correlated inversely to the efflux of BSA; the deeper the ingress of glutaraldehyde, the less noncross-linked protein is free for diffusion. The various depths of penetration can be roughly compared visually, either by noting the thickness of the fixed portions by the differential colors of the intact gel or the thickness of the remaining wafer following boiling to remove the collagen. However, the authors have chosen to use biochemical data instead of physical measurements. Thus, while this assay does not provide linear descriptions of the degree of penetration, they are confident that it indirectly, and more accurately, measures the same parameter.

Penetration of glutaraldehyde into the gels was clearly a function of concentration; increased exposure at any given concentration did not enhance the depth of fixation significantly. The results also suggest that glutaraldehyde penetration is self-limiting, tending to reach a common maximum despite ever-increasing concentrations. This conclusion is based on the finding that the secondary diffusion of BSA was decreased by approximately the same amount by the 2, 4, and 8% solutions. These results should not be interpreted that time has no bearing on glutaraldehyde fixation. To the contrary, the

previous experiment definitively demonstrated that longer periods of incubation effect greater cross linking. What these assays are suggesting is that while the quality of fixation can be enhanced by longer contact and stronger solutions, the depth of penetration can only be increased by the latter. In addition, it would appear that only a limited zone of fixation will result, regardless of the concentration of glutaraldehyde.

Two observations suggest that these findings will be verified by in vivo analysis. First, it has long been known by electron microscopists that tissues must be cut into small pieces in order to ensure complete glutaraldehyde fixation. Second, the histologic studies of glutaraldehyde-treated human primary teeth by Kopel et al. (1980)
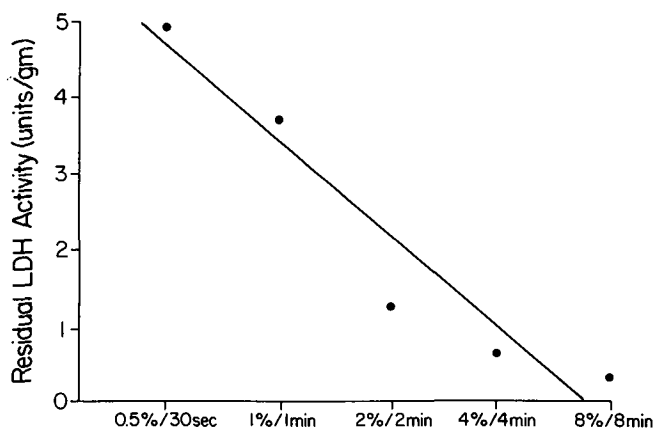

Fig 6. Graph of residual LDH activity plotted against log of 5 selected pairings of concentration and treatment periods to illustrate additive effect of time and concentration. The r value of the regression analysis was 0.95.

demonstrated a minimal zone of fixed pulp tissue. Thus, in the classic pulpotomy, where affected and infected coronal tissue is removed, leaving healthy radicular tissue, glutaraldehyde appears to be an ideal agent. Its sphere of influence is profound but localized.

While the studies with gel revealed characteristics of protein fixation in a restricted environment, the authors wanted to test the response of pulp tissue to several concentrations and treatment periods. For this purpose, they selected the enzyme LDH which previously has been shown to be a sensitive indicator of biological fixation (Mejare et al. 1976). Their results demonstrated that LDH is sensitive to both increasing concentrations and treatment periods. To be precise, LDH inhibition vs. concentration at any treatment period exhibits characteristics of a log dose response. Practically, this means that doubling the concentration of glutaraldehyde will not double the effect. However, since time and concentration are additive, the degree of fixation can be increased by combining stronger concentrations with longer applications. This can be seen in Figure 6 where LDH activity is plotted against the concentration/time pairings: 0.5%/30 sec; 1.0%/1 min; 2.0%/2 min; 4%/4 min; and 8%/8 min. The curve exhibits strong log dose characteristics

also ($r = 0.95$), and the negative slope is greater than those of the other curves (Fig 5). This finding suggests that the most efficient protocol to enhance fixation should incorporate stronger concentrations.

If enzyme inhibition as a marker for general biologic inactivation is accepted, glutaraldehyde should nullify lysosomal and other autolytic enzymes capable of tissue destruction and inflammation. This bifunctional reagent apparently fixes tissue profoundly, but in a very localized way. Previous research has indicated that the reaction products of glutaraldehyde are not very antigenic (Ranly et al. 1985). Taken altogether, the authors feel that glutaraldehyde will make an ideal pulpotomy agent: it should fix the coronal radicular pulp well (sealing the tissue from the influence of restorative materials), penetrate a shallow distance, and do little to provoke an inflammatory reaction. Although the ideal situation would be a bridge of reparative dentin, an unprovocative seal of fixed tissue might be the next best thing.

Realizing that extrapolation from in vitro studies to real life situations should be exercised with care, some clinical guidelines are suggested by this study. First, buffering the glutaraldehyde amplifies its cross linking and presumably decreases the harshness of the acidity. Second, the authors do not feel that the best clinical results will be obtained with the use of a 2% solution with an application time of 5 min. They anticipate greater success with the use of 4% glutaraldehyde applied for 4 min or a 2-min treatment with an 8% preparation.

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