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Cytotoxicity of glutaraldehyde and formaldehyde in relation to time of exposure and concentration

Hsiao Wen Sun, BDS, MS Robert J. Feigal, DDS, PhD
Harold H. Messer, MDSc, PhD

Abstract

The interacting effects of time of exposure and concentration as factors in cytotoxicity were compared for glutaraldehyde and formaldehyde. Cells from a human fibroblast cell line (WI-38) grown to confluence in 24-well trays were exposed to a range of concentrations of each agent, for periods of 4 to 24 hr. Cytotoxicity was measured by its effects on mitochondrial dehydrogenase activity, as assayed biochemically. Cytotoxic effects of formaldehyde occurred over a narrow concentration range from nontoxic to maximally toxic, and the range was little affected by time of exposure. In contrast, glutaraldehyde exerted its effect over a wider concentration range, and longer exposure times were necessary for maximal toxicity. The data suggest that long contact times of glutaraldehyde with dental pulp are necessary for maximum fixation. While 19% formaldehyde appeared to be more toxic than 2.5% glutaraldehyde in terms of serial dilution, little difference in cytotoxicity was observed when the data were calculated in terms of molar concentrations of the two agents.

Introduction

Glutaraldehyde has emerged as a prospective substitute for formocresol as a pulpotomy agent (s'-Gravenmade 1975), since potential problems with mutagenicity and systemic spread of formocresol have been emphasized increasingly (Myers et al. 1978; Lewis and Chestner 1981). Glutaraldehyde has been evaluated using a variety of experimental approaches, including cytotoxicity measurements (Seow and Thong 1986; Jeng et al. 1987), animal pulpotomy studies (Davis et al. 1982; Tagger and Tagger 1984), and limited clinical trials in humans (Kopel et al. 1980; García-Godoy 1983, 1986; Fuks et al. 1986). Overall, glutaraldehyde appears to be more acceptable biologically than formocresol, in that its higher molecular weight and two active aldehyde groups limit its tissue penetration (Dankert et al. 1976; Lekka et al. 1984; Tagger et al. 1986), and hence, reduce the extent of inflammatory response (Davis et al. 1982; Weemes et al. 1982; Tagger and Tagger 1984).

In a previous study comparing cytotoxicity of formocresol and glutaraldehyde in human pulp fibroblasts (Jeng et al. 1987), 2.5% glutaraldehyde was found to be 15-20 times less toxic than formocresol or 19% formaldehyde. In that study, 19% formaldehyde was found to exert the same cytotoxic effect as formocresol. Thus, in the present study, formaldehyde was used rather than formocresol, to allow a direct comparison of the two aldehydes. We also observed that longer exposure to glutaraldehyde was necessary to exert the maximum cytotoxic effect, and suggested that this may have clinical implications in terms of contact time of the pulpotomy agent with the pulp (Jeng et al. 1987).

In this study we have investigated further the effect of exposure time on the apparent cytotoxicity of glutaraldehyde and formaldehyde. As an alternative to dye uptake as a measure of cytotoxicity, which is a nonspecific marker of cell membrane damage, we have used enzyme cytochemistry to measure the extent of inhibition of mitochondrial dehydrogenase activity (Mosmann 1983; Tyas 1988). Since mitochondria are the main site of cellular respiration, effects of toxic agents on mitochondrial enzyme activity may be a more sensitive and specific indicator of toxic effects on the cells. This approach, which measures enzyme activity without disrupting the cell monolayer, lends itself very readily to assessing the effects of different incubation times and concentrations of cytotoxic agents.

Materials and Methods

Cell Cultures

WI-38 (human embryonic lung) fibroblasts, obtained from the American Type Culture Collection, Rockville, MD, were used as surrogates for pulp cells. Cells are grown in Dulbecco's Modified Eagle Medium (DMEM — Gibco Laboratories, Life Technologies Inc., Grand

Island, NY) with L-glutamine, supplemented with fetal bovine serum (10%) and penicillin-streptomycin solution (100 U/ml penicillin, 100 µg/ml streptomycin final concentration), and incubated in 95% air/5% CO_2 at 37°C. Cells were harvested from flasks using trypsin-EDTA mixture (Gibco Laboratories, Life Technologies Inc., Grand Island, NY), centrifuged at 600 x g, and resuspended in DMEM. Cells were then seeded into 24-well trays at a cell density of approximately $5x10^4$ cells per well and grown to confluence (three to five days).

Exposures of Cells to Glutaraldehyde and Formaldehyde

Solutions of 2.5% glutaraldehyde and 19% formaldehyde were prepared in distilled water from analytical reagent grade stock solutions. After preliminary trials to identify appropriate concentration ranges, serial dilutions of glutaraldehyde and formaldehyde were made in DMEM (plus serum) to yield final concentrations as follows:

Glutaraldehyde 2.5%
0, 0.1, 0.5, 1.0, 5.0, 10, 20, 40 µl/ml
Formaldehyde 19%
0, 0.01, 0.05, 0.10, 0.25, 0.50. 1.0 µl/ml

Duplicate wells of confluent fibroblasts were exposed to all concentrations of each agent for 4, 8, or 24 hr, and experiments were repeated three to five times, for a total of six to 10 replicates for each concentration of each medicament at all time periods. Preliminary experiments were conducted to determine whether the presence of fetal bovine serum in the culture medium would influence the response to glutaraldehyde and formaldehyde, since these agents are known to bind covalently to proteins. Metabolic activity of control cultures was reduced markedly in the absence of serum at times after 4 hr, and the response to glutaraldehyde and formaldehyde was not substantially different in the presence or absence of serum. Therefore, it was included in the medium at 10% v/v for routine cultures.

Mitochondrial Dehydrogenase Assay

Dehydrogenase activity was assayed using the MTT test of Mosmann (1983), based on the formation of a colored reaction product (formazan) from a tetrazolium salt used as the electron acceptor [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, or MTT]. The assay was conducted in the multiwell trays with the fibroblast monolayer left intact (Edmondson et al. 1988).

Following exposure of the cell layer to the appropriate concentration of the aldehyde for the required time period, the supernatant was aspirated carefully, leaving the cell layer intact in the well. One-half ml of a 0.5% solution of MTT in phosphate-buffered saline was added to each well and incubated in the dark for 4 hr at 37°C. The reaction was stopped by addition of 1 ml acid isopropanol (0.04 HCl in isopropanol), which also dissolved the colored formazan reaction product. The colored solution was assayed spectrophotometrically at 570 nm. Enzyme activity was expressed as OD units per well. Control values were typically in the range of 0.7-0.9 OD units.

Calculation of Cytotoxicity

All experimental values were calculated as a percentage of the control values (no added aldehyde) from the same multiwell tray. For each time period, data were plotted as enzyme activity vs. $\log_{10}$ concentration of aldehyde, based on an assumed log-dose response to both agents. From these log-dose response curves, two measures of cytotoxicity were derived: the maximum nontoxic concentration (below which no toxic effect was measurable); and the 50% toxic concentration (the concentration necessary to reduce enzyme activity to 50% of the control level). The concentration range of each agent tested was selected to include concentrations both below the level causing enzyme activity inhibition and above the level causing maximum inhibition. As a result, the maximum nontoxic concentration and the 50% toxic concentration were derived from regression lines drawn through only those points that included all intermediate levels of inhibition as well as the highest nontoxic concentration and the lowest maximally toxic concentration. The maximum nontoxic concentration and the 50% toxic concentration were then calculated both as dilutions of the stock solutions (expressed as µl/ml) and as molar concentrations of each agent.

Results

The inhibitory effects of glutaraldehyde and formaldehyde on mitochondrial dehydrogenase activity at each time period are shown in Figs 1 (formaldehyde) and 2 (glutaraldehyde), plotted as a log-dose response.

No inhibition of enzyme activity was observed at concentrations of 0.06 to 0.6 mM formaldehyde. The dose response curve to formaldehyde was very steep, and maximal inhibition of enzyme activity occurred at concentrations of 0.3 mM and above (Fig 1). Little effect of time of exposure to formaldehyde was noted, and the dose response curves were similar for 4, 8, and 24 hr exposure.

Toxicity of glutaraldehyde was considerably more time- and concentration-dependent (Fig 2). Even at the highest concentrations, glutaraldehyde did not achieve maximum inhibition at 4 and 8 hr, and after 24 hr the residual enzyme activity in the flat portion of the dose response curve (maximum inhibition range) was higher than for formaldehyde.

Regression analysis was performed on group mean

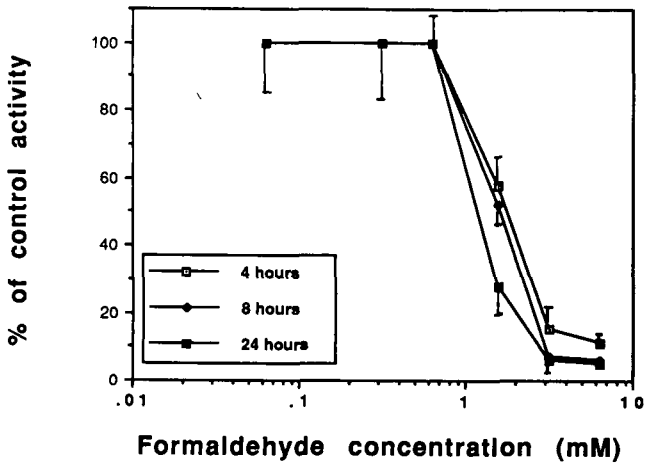

Fig 1. Time and dose response curve of mitochondrial dehydrogenase activity to formaldehyde. Each point represents the mean ± SD for nine measurements.

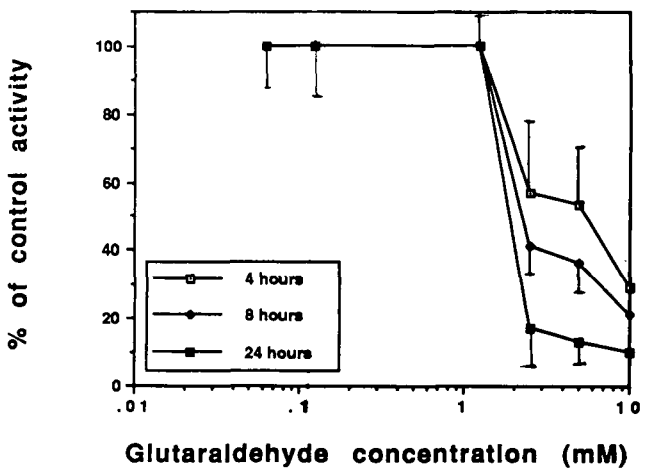

Fig 2. Time and dose response curve of mitochondrial dehydrogenase activity to glutaraldehyde. Each point represents the mean ± SD for nine measurements.

data for formaldehyde and glutaraldehyde at each time period, to yield estimates of the maximum nontoxic concentration and the 50% toxic concentration (Table). For formaldehyde, neither value was influenced markedly by time of exposure, and the 24-hr values were within approximately 80-90% of the corresponding 4-hr values. In addition, the ratio of 50% toxic concentration to maximum nontoxic concentration was small, averaging approximately 2.5:1 for all time periods. For glutaraldehyde, the maximum nontoxic concentration was little affected by time of exposure (Table), but the 50% toxic concentration declined by more than 50% between 4 and 24 hr. The ratio of 50% toxic concentration to maximum nontoxic concentration was greater than for formaldehyde at 4 hr (5:1), but declined with increased time of exposure. After 24-hr exposure to glutaraldehyde, the ratio was comparable to that of formaldehyde (2.5:1).

Table 1. Maximum Nontoxic and 50% Toxic Concentrations of Formaldehyde and Glutaraldehyde

	4 hr	*8 hr*	*24 hr*
19% formaldehyde			
Maximum nontoxic concentration	0.104 µl/ml*	0.096 µl/ml	0.091 µl/ml
	0.66 mM†	0.61 mM	0.58 mM
50% toxic concentration	0.269 µl/ml	0.227 µl/ml	0.212 µl/ml
	1.70 mM	1.44 mM	1.34 mM
2.5% glutaraldehyde			
Maximum nontoxic concentration	3.91 µl/ml	4.11 µl/ml	3.40 µl/ml
	0.98 mM	1.03 mM	0.85 mM
50% toxic concentration	19.31 µl/ml	12.15 µl/ml	8.35 µl/ml
	4.83 mM	3.04 mM	2.09 mM

* Expressed as dilution of original strength formaldehyde or glutaraldehyde.

† Expressed as molar concentration of the agent.

The toxicity of glutaraldehyde was much less than that of formaldehyde when calculated as dilution of the stock solutions (i.e., in µl/ml). Expressed in terms of maximum nontoxic concentration, 19% formaldehyde was approximately 40 times more toxic than 2.5% glutaraldehyde at all times of exposure. In terms of the 50% toxic concentration, formaldehyde ranged from 70 times more toxic after 4 hr of exposure to 40 times more toxic after 24 hr. When the data are calculated as molar concentrations, however, the differences become much smaller (Table), and the toxicity of formaldehyde was generally less than twice that of glutaraldehyde.

Discussion

Using an enzyme assay for evaluating cytotoxicity provides a more quantitative approach than more commonly used techniques such as dye exclusion or uptake (Guess et al. 1965; Tronstad et al. 1978; Stanford 1980), and the assay is able to detect any degree of enzyme inhibition. The MTT assay used here is technically simple, especially since the enzyme assay is conducted in the multiwell tray without disruption of the cell monolayer (Mosmann 1983; Edmondson et al. 1988). Since the MTT test involves mitochondrial function, concern may arise that the test will underestimate the effects of agents that bind to the cell membrane and do not penetrate the cell readily. Glutaraldehyde falls into this category (Munton and Russell 1970). Nonetheless the MTT assay relies on an intact cellular respiratory system. Agents that affect cell membrane function also will affect mitochondrial dehydrogenase activity (Mosmann 1983), and therefore, will be detected with the MTT assay.

Many different cell types have been used for cytotoxicity studies. Ideally, using a human target cell that is affected by the agent in normal clinical use is highly desirable (Rounds 1978). While human pulp fibroblasts in primary culture have been used in some studies (Das 1981; Feigal et al. 1985; Messer and Feigal 1985; Jeng et al. 1987), they are difficult to culture (Das 1981). For this study we used WI-38 cells, of human embryonic lung fibroblast origin, as an alternative diploid cell line. Formaldehyde (19%) was used in this study rather than formocresol, since in a previous study we found that the cytotoxic effects of the two were identical, while those of cresol were much lower (Jeng et al. 1987). The maximum nontoxic concentrations of glutaraldehyde and formaldehyde that we observed were two to four times higher than those reported previously using human pulp fibroblasts (Jeng et al. 1987).

The relative toxicities of formaldehyde and glutaraldehyde are very similar when calculated as molar concentrations. This was especially true at 24 hr, when glutaraldehyde had had time to exert its maximal (or near maximal) effect. Both the maximal nontoxic and 50% toxic concentrations were only approximately 50% greater for glutaraldehyde than for formaldehyde. In our previous study (Jeng et al. 1987), very little difference in toxicity was also observed when the data were calculated in terms of molarity rather than dilution.

Concentration and time of exposure showed a strong interaction in the cytotoxicity of glutaraldehyde, but were much less critical in the toxicity of formaldehyde. For both agents, the maximal nontoxic concentration was little affected by exposure time in the range of 4 to 24 hr. The 50% toxic concentration of formaldehyde was only approximately 2.5 times the maximum nontoxic concentration, with little change between 4 and 24 hr. In contrast, the 50% toxic concentration of glutaraldehyde was five times the maximum nontoxic concentration after 4 hr of exposure, but declined by more than one half between 4 and 24 hr, from 4.83 mM to 2.09 mM. Ranly et al. (1987) also observed substantial time and concentration effects of glutaraldehyde, as measured by the fixation of collagen-BSA gels and the inhibition of enzyme activity in bovine pulp fragments.

The increasing toxicity of glutaraldehyde with increasing time of exposure may result from slow death of cells after the initial insult, rather than from a continuing effect of exposure to glutaraldehyde. Our experimental protocol did not allow a clear distinction between these two possibilities. If the increasing toxicity did result from slow death of cells, then the apparent maximum nontoxic concentration would be expected to decline with increasing time of exposure. We found little change with time in the maximum nontoxic concentration, and thus conclude that the effect of glutaraldehyde on cells is a progressive one, based on duration of exposure.

Neither the serial dilution of agents nor the times of exposure that we used in this study closely simulates an actual pulpotomy procedure. Clinically, a much stronger concentration of the agent is applied to the pulpal surface for a much shorter time period. Nevertheless, the time of contact of glutaraldehyde with pulp tissue is clearly an important element in its action on the pulp. Contact time should be as long as possible to ensure complete fixation (assuming that enzyme denaturation can be equated with fixation). Ranly et al. (1987) suggested that a higher concentration of glutaraldehyde applied for a shorter time period may circumvent the problem (e.g. 4% for 4 min or 8% for 2 min, rather than 2% for 5 min).

This approach raises a number of concerns. With shorter exposure times, the gradient of glutaraldehyde concentration in the pulpal tissue will be steeper, so that the quality of fixation may not be adequate except at the very surface of the pulpotomy site. While a narrow zone of adequate fixation may be achieved, partial cell damage deeper to this zone may lead to chronic cell injury. Fixative and antibacterial functions are correlated. Therefore, a superficial fixation may result in insufficient depth of antibacterial action. Systemic distribution may also be increased as a result of the application of a greater quantity of the agent.

Clearly, time and concentration factors have important clinical implications, and much more work remains to be done before precise clinical guidelines for the use of glutaraldehyde as a pulpotomy agent can be established.

Dr. Sun is resident, division of endodontics; Dr. Feigal is associate professor, division of pediatric dentistry; and Dr. Messer is professor, division of endodontics. All are at the University of Minnesota School of Dentistry, Minneapolis, MN.

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