Comparison of the cytotoxicity of formocresol, formaldehyde, cresol, and glutaraldehyde using human pulp fibroblast cultures

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Abstract

Serial dilution and agar overlay techniques were used to compare the cytotoxicity of formocresol, 19% formaldehyde, 35% cresol, and 2.5% glutaraldehyde to human pulp fibroblasts. The maximum nontoxic concentration of each agent was determined to allow quantitative comparisons both of the agents tested and of the techniques used. Formaldehyde was found to be the major component of formocresol which is responsible for the cytotoxic effect on human pulp fibroblasts. Two and one-half per cent glutaraldehyde was 15-20 times less toxic than either formocresol or 19% formaldehyde. Cresol measured 40 times less toxic than formaldehyde or formocresol. Both serial dilution and agar overlay techniques appear to be sensitive and effective methods for testing the toxicity of diffusible agents.

Despite the clinical success of pulpotomies performed using formocresol as the active agent, several authors have raised questions about continued use of the drug. These questions are supported by reports of systemic spread of formaldehyde after pulpotomy (Myers et al. 1978), adverse effects on the enamel of succedaneous teeth (Pruhs et al. 1977), and the mutagenic and carcinogenic potential of formocresol in animals (Muller et al. 1978).

Morawa et al. (1975) have recommended a 1:5 dilution of formocresol with glycerine and water for pulpotomies. This recommendation is based on the study by Loos et al. (1973) which showed that formocresol in full concentration, while effective in developing cytostasis, may produce irrecoverable damage to connective tissue. On the other hand, a 1:5 dilution of formocresol, which creates metabolic effects similar to those achieved by full strength formocresol, produces an earlier recovery of cellular respiratory enzyme activities in connective tissues (Loos et al. 1973)

s'-Gravenmade (1975) proposed that glutaraldehyde could be used as a new pulpal fixative in dentistry. Glutaraldehyde has been considered a possible substitute for formocresol because: (1) it is a more active

fixative agent, cross linking proteins by virtue of its two active sites (Russell 1976); (2) its tissue penetration is limited (Tagger et al. 1986); and (3) the zone of infiltration is more restricted following application to exposed pulps (Davis et al. 1982). Although one investigator reported favorable clinical results with glutaraldehyde (Garcia-Godoy 1983), little information is available concerning tissue response to glutaraldehyde as a pulpotomy agent.

Very few toxicity studies of glutaraldehyde have been conducted. Seow and Thong (1986) examined the effects of pulpotomy medicaments (glutaraldehyde, formocresol) on polymorphonuclear leukocyte (PMN) adherence, because they considered that the persistent activation of PMNs by pulpotomy medicaments may contribute to the chronic inflammatory changes and root resorption seen in histologic sections. The results showed that formocresol caused lysis of PMNs at high concentrations, but activation of PMN adherence at low concentration. By contrast, glutaraldehyde did not produce PMN lysis at high concentrations, nor did it cause activation of PMN adherence at low concentration.

Clearly, the toxicity of formocresol has been investigated very intensively and the potentially harmful effects to humans have been discussed by others. Very few studies of the toxicity of glutaraldehyde have been done, and none of them were cytotoxicity studies. Toxicity testing using cells in culture is an efficient and effective method to evaluate cellular effects of agents. Both glutaraldehyde and formaldehyde are diffusible substances, and so are suitable for testing by serial dilution and agar overlay techniques on cells in vitro. The authors of this study chose human pulp fibroblasts as the test cells, because they are derived from the tissue which would be in contact with a pulpotomy agent. This study compared the cytotoxicity of formocresol, each of the formocresol constituents, and glutaraldehyde. An attempt at improved quantitation of toxicity was made in this study by measuring the maximum

concentration achieved at any point in an agar overlay diffusion system and relating this to the damage observed in the cells. A third component of the study was to compare the sensitivity of the serial dilution technique and the agar overlay technique.

Materials and Methods

Pulpotomy Agents

The toxic effects of formocresol, its separate constituents formaldehyde and cresol, and glutaraldehyde were tested. Formulations of the different agents were prepared in the laboratory according to the composition of the different materials as used clinically. Chemicals used were of reagent-grade quality obtained from chemical supply companies.

1. Formocresol - mixed according to Buckley's formula (1904): 19% formaldehyde, 35% cresol,[a] in a vehicle of 15% glycerine and water.
2. 19% Formaldehyde - diluted from 37% formalin with distilled water.
3. 35% Cresol - diluted from 100% m-cresol with 95% ethanol. (Cresol could not be dissolved in water or a glycerine-water vehicle without rapid phase separation.)
4. 2.5% Glutaraldehyde - diluted from 50% glutaraldehyde solution, with distilled water.

Cell Cultures

Primary cell cultures of human pulp fibroblasts (derived from third molar pulps) were used (Feigal et al. 1985). Cells were grown in Dulbecco's Modified Eagle Medium[b] (DMEM) containing L-glutamine with 10% fetal bovine serum and penicillin-streptomycin as antibiotics, using standard culture conditions of 37°C and an atmosphere of 5% CO_2 in air.

Serial Dilution Technique

Cells were detached from the flasks using trypsin harvesting methods and seeded into 24-well trays[c] (5 x 10^4 pulp fibroblasts per well). A confluent layer (Fig 1) was achieved within 4-5 days. A 1% agar medium solution was prepared by mixing equal volume of 2%
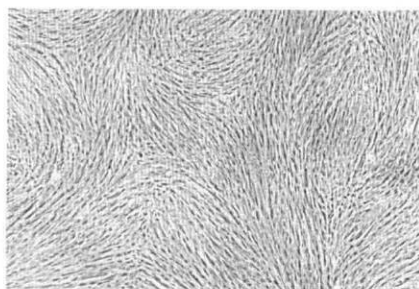

FIG 1. A confluent layer of pulpal fibroblasts (original magnification 100x).

agar and DMEM, and maintained at 45-48° C in a water bath until use. The four tested medicaments were serially diluted with the prepared 1% agar medium. Four 24-well trays were used for each replicate of the experiment, one tray per medicament, and at least two wells for each concentration. Formocresol (1 µl/ml) served as a positive (toxic) control and distilled water as a negative (nontoxic) control. All the trays were incubated for 24 hr after the agar was solidified.

The neutral red vital staining technique as well as morphological criteria were used to assess cell damage. A fresh, neutral red staining solution[d] was prepared before testing by diluting the stock neutral red solution (1% in water) by 1:100. The monolayer was stained by applying 1 ml neutral red to the surface of the agar of each well. Excess neutral red was aspirated after 30 min. The flasks were incubated for another hour in the dark and the results were read under an inverted microscope.

Agar Overlay Technique

Preparation of Medicaments

1. ^{14}C-labeled paraformaldehyde[e] was mixed with 37% formaldehyde solution to yield an activity of 250 µCi/ml in either formocresol or 19% formaldehyde.
2. Glutaraldehyde [[14]C-(1,5) glutaraldehyde][f] was added to a 2.5% aqueous solution of glutaraldehyde to yield an activity of 250 µCi/ml.
3. Cresol (uniformly labeled ^{14}C-p-cresol)[e] was dissolved in unlabeled cresol to an activity of 500 µCi/ml. The cresol was then diluted to a 35% solution.

The agar overlay technique used in these studies was a slight modification of the procedure described previously (Guess et al. 1965; Messer and Feigal 1985). Pulp fibroblasts were grown to confluence in 60 mm Petri dishes. Cells then were overlaid by 5.0 ml 1% agar medium, yielding a layer approximately 2.0 mm thick. After the agar had set, a central well 1.0 mm in diameter was punched in the agar. A 2.0-mm length of an extra-coarse endodontic paper point 1.0 mm in diameter to which 2.0 µl of test medicament had been added was inserted into the central well so that the top of the paper point was flush with the surface of the agar. Under these conditions, the paper point was in close contact with the agar, permitting ready diffusion of the medicament into the agar. Triplicate plates were incubated for four, eight, 12, or 24 hr. At each time period, duplicate agar plugs (1.0 mm in diameter and 5.5 µl in volume) were

taken at radii of 20, 15, 10, 7, and 3 mm from the central well to measure the extent of diffusion of the medicament. Each plug was added to 12 ml scintillation fluid in a scintillation vial for liquid scintillation spectrometry. After the plugs were taken, the staining technique was completed and the radius of the zone of cell damage was measured under the inverted microscope.

The concentration of medicament at any distance from the center well at any time period then could be calculated from the volume of the sample plug and the radioactivity in the sample. A series of curves was generated showing the concentration of medicament at different distances from the center well at varying times. The maximum nontoxic concentration then could be determined from these curves, based on the radius of the zone of cell damage.

Evaluation of Cytotoxicity

Cytotoxity was evaluated using an inverted microscope with phase contrast at 100x magnification and was based on two criteria: staining of the cells and cell morphology.

In the serial dilution technique, all cells within each well were contacted with a homogeneous concentration of medicament. All concentrations of a medicament were represented in duplicate on one 24-well tray. The authors defined their scoring system as: no stain = 0 (Fig 2a); partial stain = 1 (Fig 2b); and normal stain = 2 (Fig 2c). So the mean score at a given concentration is equal to $(0 \times n_1 + 1 \times n_2 + 2 \times n_3)/(n_1 + n_2 + n_3)$ where n_1 = number of no staining wells, n_2 = number of partial staining wells, and n_3 = number of normal staining wells. The maximum nontoxic concentration will be the concentration equal to a mean score of 2 (i.e., maximum concentration causing no cell damage).

In the agar overlay technique, cell damage was assessed using an inverted microscope. Four readings were made on each Petri dish by following quadrant radii from the center of the disc, where the medicament was placed, to a point where normal neutral red-staining and normal cell morphology occurred in 100% of cells in the field. The zone of cell damage was derived

from the mean of the four radii expressed to the nearest whole millimeter.

Results

Serial Dilution Technique

Pulp fibroblast response to different concentrations of 2.5% glutaraldehyde is shown in Table 1. This table is representative of the scores for each medicament

TABLE 1. Scoring from Staining Results of 2.5% Glutaraldehyde in Serial Dilution Technique

Concentration (µl/ml agar)	No. of wells assigned a score of			Mean Score
	0*	1**	2***	
10.0	8	0	0	0.0
5.0	5	3	0	0.4
2.5	1	5	2	1.1
1.0	1	3	8	1.6
0.5	0	0	8	2.0
0.1	0	0	8	2.0

* No stain, ** Partial stain, *** Normal stain.

tested and shows the degree of staining for each well as observed from four experiments, each experiment measuring 2-4 replicates. Concentrations of 2.5% glutaraldehyde of 10 µl/ml agar and greater were completely toxic to cells, while concentrations of 0.5 µl/ml and less were nontoxic as judged by staining response. Mean scores for each concentration of medicament were derived as described in the Materials and Methods section. Graphic interpolation yielded a maximum nontoxic concentration of 0.65 µl/ml (Fig 3, page 298).

The final estimation of maximum nontoxic concentrations of four pulpotomy agents each derived as illustrated with glutaraldehyde (Fig 3) is shown in Table 2 (page 292). The results show that the maximum nontoxic concentrations of formocresol and 19% formaldehyde are both 0.051 µl/ml; 35% cresol is 0.91 µl/ml, which is much less toxic than 19% formaldehyde. It is obvious that the major toxic substance of formocresol is formaldehyde. Comparing the cytotoxicity of formocresol and 2.5% glutaraldehyde shows that full strength formocresol is 13 times more toxic than 2.5% glutaraldehyde.
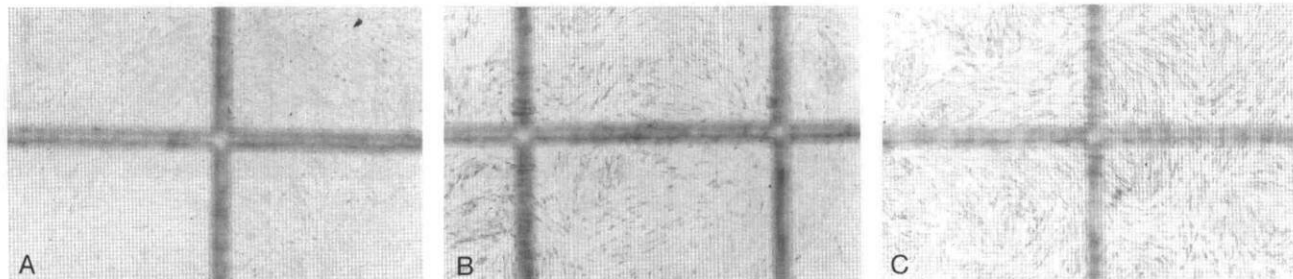

FIG 2a *(left)*. Neutral red vital stain: no stain. The dark line shown is the grid line at the bottom of the Petri dish. **Fig 2b** *(center)*. Neutral red vital stain: partial stain. The dark line shown is the grid line at the bottom of the Petri dish. **Fig 2c** *(right)*. Neutral red vital stain: normal stain. The dark line shown is the grid line at the bottom of the Petri dish. Each unit is two mm in length (original magnification 100x).
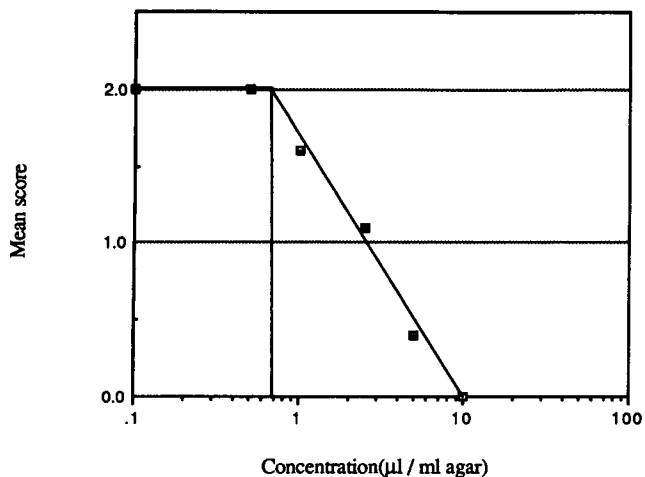
Concentration(μl / ml agar)

FIG 3. Cytotoxicity of 2.5% glutaraldehyde in serial dilution technique. Each point represents the mean score at a given concentration of glutaraldehyde: the maximum nontoxic concentration was derived from the mean score at 2.

Agar Overlay Technique

Measurement of concentration of each test substance was performned at different radii._Representive results of the glutaraldehyde measurement are shown. Following the placement of 14C-glutaraldehyde in the center well, the concentration at increasing distances from the center was determined at intervals up to 24 hr. Figure 4 shows the extent of diffusion at each time interval.

Assessment of cell damage was performed by neutral red vital staining technique after each time period.
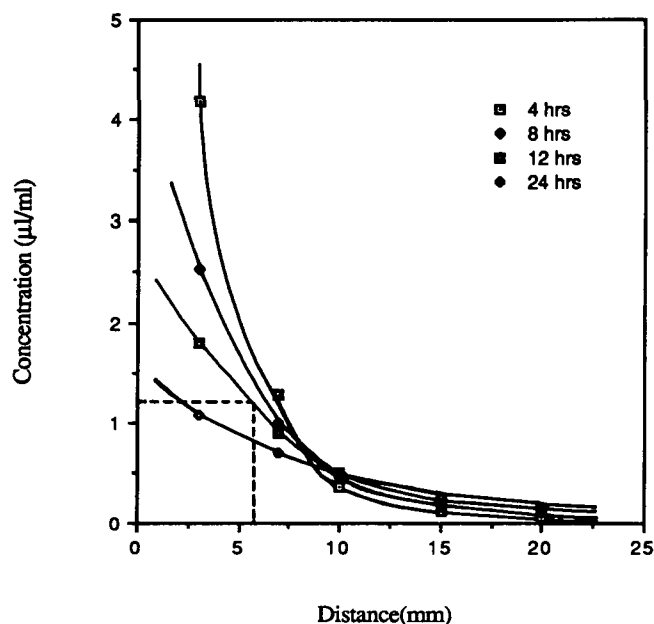

Distance(mm)

FIG. 4 Concentration curve for ¹⁴C-glutaraldehyde. This graph shows the maximum concentration achieved at each distance from the center at any time. Conversion to μl glutaraldehyde/ml agar was based on the known activity of the ¹⁴C-glutaraldehyde and the volume of the agar plug taken for analysis.

TABLE 2. Maximum Non-Toxic Concentrations of Pulpotomy Agents in Serial Dilution Technique

Pulpotomy Agents	Maximum Non-Toxic Conc.* (μl/ml)
Formocresol	0.051
19% Formaldehyde	0.051
35% Cresol	0.91
2.5% Glutaraldehyde	0.65

* Defined as the maximum concentration producing a mean score of 2 as estimated from the cytotoxicity graph.

TABLE 3. Maximum Non-Toxic Concentration of Pulpotomy Agents in Agar Overlay Technique

Pulpotomy Agents	Maximum Non-Toxic Conc. (μl/ml)
1:5 formocresol	0.26*
Full strength formocresol	0.052
1:5 19% formaldehyde	0.26
Full strength 19% formaldehyde	0.052
35% cresol	2.10
2.5% glutaraldehyde	1.20

* All the concentrations are estimated from the concentration-radius graph.

A pilot study showed that two μl of full strength formocresol killed all cells in the Petri dish within four hr. Therefore, instead of full strength, 1:5 dilutions of ¹⁴C-formocresol and of 19% ¹⁴C-formaldehyde were used in all dishes tested. The zones of damage were recorded after each time period.

From the concentration curve and the zone of cell damage, the maximum nontoxic concentration of each pulpotomy agent was estimated (Table 3). For both formocresol and 19% formaldehyde the maximum nontoxic concentration was estimated to be 0.052 μl/ml (which was derived from the maximum nontoxic concentration of 1:5 dilution of formocresol and 19% formaldehyde), based on the concentration which was achieved at the 20-25 mm radius at times greater than 4 hr. The maximum nontoxic concentration of 35% cresol was 2.10 μl/ml, which is 40 times less toxic than 19% formaldehyde. The cytotoxicity of 2.5% glutaraldehyde was intermediate between 19% formaldehyde and 35% cresol, being 20 times less toxic than 19% formaldehyde and twice as toxic as 35% cresol.

Comparison of Serial Dilution and Agar Overlay Techniques

The actual maximum nontoxic concentrations of four pulpotomy agents estimated from the serial dilution technique and the agar overlay technique are compared in Figure 5 (page 299).

Both techniques show the cytotoxicity of pulpotomy agents to pulp fibroblasts in the following sequence: formocresol and 19% formaldehyde are more toxic than 2.5% glutaraldehyde, and 2.5% glutaraldehyde is more toxic than 35% cresol. The major toxic constituent in

formocresol is formaldehyde and not cresol.

In general, the maximum nontoxic concentrations estimated from the agar overlay technique were similar to those estimated from the serial dilution technique. The values derived for formocresol and formaldehyde were almost identical using the two techniques, while the agar overlay technique gave higher values for cresol and glutaraldehyde (approximately double the serial dilution values).

Discussion

In this study, the serial dilution technique and agar overlay technique were chosen to test the diffusible materials formocresol, its separate constituents formaldehyde and cresol, and glutaraldehyde. The agar overlay procedure depends on the diffusion of the test material to the target cells through a layer of agar. The authors attempted to get a better quantitative measure of cytotoxicity by modifying the conventional agar overlay technique to allow the measurement of the concentration of the medicament at any point. The modification involved applying radiolabeled material in the center well, taking plug samples at different distances from the center well at different time periods, and calculating the concentration of the medicament after diffusion. A potential problem arose in this measurement: the maximum concentration of medicament was not achieved throughout the entire plate at the same time, requiring multiple measurements. By measuring the concentration at increasing distances over many time intervals, a composite curve could be constructed to yield the maximum concentration achieved at any distance from the center well. As would be predicted, the maximum concentration was reached sooner for small diameters, and subsequently declined as the
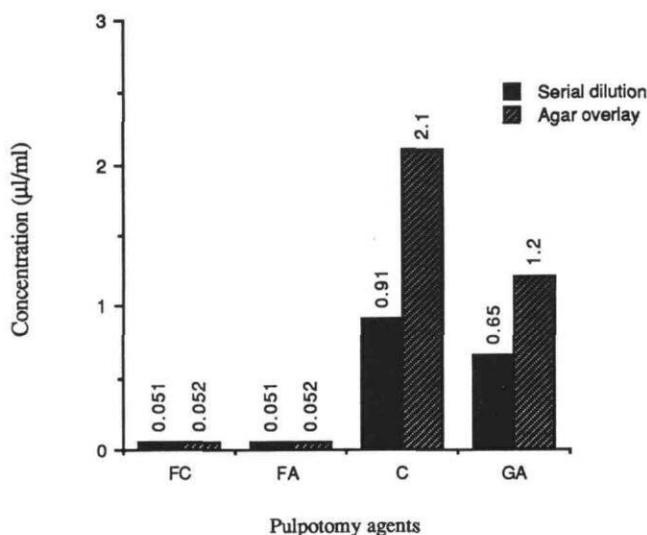

FIG 5. Comparison of maximum nontoxic concentrations of pulpotomy agents between the serial dilution technique and the agar overlay technique.

medicament continued to diffuse outward.

The results showed that the two techniques gave similar results in terms of maximum nontoxic concentration of the different medicaments. The values were identical for formocresol and formaldehyde, but lower for cresol and glutaraldehyde in the serial dilution technique. There is a tendency for the serial dilution technique to underestimate maximum nontoxic concentration compared to the agar overlay technique; using the serial dilution technique to detect such a narrow range of critical concentrations would require several more experimental runs of narrower dilution ranges to achieve the same precision as the agar overlay technique.

Another benefit of using the agar overlay technique is that this technique involves both cytotoxicity and diffusibility of the test medicaments. This more closely simulates the clinical situation in that pulpotomy agents will fix the pulp tissue only after they diffuse out. In contrast, technically the serial dilution technique is much easier to use. The medicament is homogeneously distributed throughout the agar, and directly contacts the target cells at a known concentration. Fewer cells are needed for each experiment because testing each concentration in a small well is enough to get the necessary information. For the most precise measurement of maximum nontoxic concentration, however, several experiments must be run consecutively, with each run narrowing the range of concentrations tested.

The results of this study agree with that of Massler and Mansukhani's study (1959) in that they determined that cresol was less toxic than formaldehyde, although they used 7.4% formaldehyde and 100% cresol in contrast to the 19% formaldehyde and 35% cresol in Buckley's formula. In this experiment, both the serial dilution technique and the agar overlay technique showed that the cytotoxicity of formocresol and formaldehyde to pulp fibroblasts is equal and that each is much more toxic than 35% cresol. Ranly and Fulton (1976), in a rat histological study, found cresol to be a more caustic ingredient than formaldehyde in formocresol because of delayed recovery in the cresol group compared to the formaldehyde-treated group.

Very few toxicity studies of glutaraldehyde have been done. Seow and Thong (1986) examined PMN adherence after contact with glutaraldehyde and formocresol. They concluded that glutaraldehyde was less toxic than formocresol. The results of the present study are similar to those of Seow and Thong. In the serial dilution technique, full strength formocresol was 13 times more toxic than 2.5% glutaraldehyde. In the agar overlay technique, even a 1:5 dilution of formocresol was still more toxic than 2.5% glutaraldehyde. The results confirm the findings of Loos et al. (1973) that applying a 1:5 dilution of formocresol decreases the cytotoxicity of formocresol to pulp fibroblasts. In the

present study, a 1:5 dilution of formocresol was only 2-3 times more toxic than 2.5% glutaraldehyde.

If reduced toxicity is a consideration in assessing 2.5% glutaraldehyde as a substitute for formocresol as a pulpotomy agent, it is clear from the data that a 1:5 dilution of formocresol decreases the toxic effect of this agent to a level approximately comparable to the cytotoxicity of 2.5% glutaraldehyde. To further address this issue, it might be necessary to do a similar study to that of Loos et al. (1973): dilute 2.5% glutaraldehyde to find a maximum dilution that preserves the metabolic effects of full strength 2.5% glutaraldehyde, but decreases cytotoxicity to the minimum.

Another finding of this study with potential clinical implications is that glutaraldehyde diffused more slowly and needed a longer time to achieve the maximum toxic effect than formocresol or formaldehyde. Further investigation needs to be done to determine if glutaraldehyde may need longer contact time with pulp tissue in a clinical setting to achieve the best fixation .

In summary, two in vitro cytotoxicity tests using human pulp fibroblasts show that formaldehyde is the most toxic constituent of formocresol and that 2.5% glutaraldehyde is 15-20 times less toxic than both formocresol or 19% formaldehyde.

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