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Research Article

Investigating the Mutagenic Effects of Three Commonly Used Pulpotomy Agents Using the Ames Test

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Abstract

Purpose: The mutagenic potency of materials used in dentistry is of great concern. The Ames test is a bacterial reverse mutation assay, which is used to determine the mutagenicity potential of chemicals. In this study, the Ames test was used to compare mutagenic effects of three pulpotomy agents, namely, CEM cement, formocresol and ferric sulfate.

Methods: TA100 strain of *Salmonella typhimurium* was used to evaluate mutagenicity of different concentrations of pulpotomy materials in the presence and absence of enzymatic system found in rat liver S9 fraction. Negative controls were 1% dimethyl sulfoxide and water. The positive controls were sodium azide and 2-aminoanthracene. The number of colonies per plate was counted. The material was regarded mutagenic if the number of histidine revertant colonies was twice or more than the spontaneous revertant colonies (Ames mutagenicity ratio).

Results: Ferric sulfate was found mutagenic in the concentrations prepared by addition of 50 µL of its 1 in 100 and 1 in 1000 times diluted solutions to the culture medium in the absence of S9 fraction (Ames test ratios of 2.8 and 2.2, respectively). Formocresol showed strong toxicity toward TA100 strain of *S. typhimurium* up to the concentration as low achieved using 1000 times diluted solution of the original preparation, particularly in the presence of S9 fraction. Ames assay failed to detect significant reverse mutations in all the concentrations of CEM cement.

Conclusion: In contrast to formocresol and ferric sulfate, CEM cement is a less toxic and non-mutagenic agent.

Introduction

In spite of advancements in modern dentistry and the growing importance of tooth preservation, most of the primary and permanent teeth are still lost early, resulting in several malocclusion and aesthetic problems.[1,2] Therefore, preservation of teeth and their surrounding tissues is an important and fundamental issue.[3] Aiming to preserve the vitality of the pulp, vital pulp therapy (VPT) is a common treatment in pediatric dentistry and endodontics.[4] Numerous materials are used for VPT, including calcium hydroxide, zinc oxide eugenol, formocresol, ferric sulfate, adhesives, enamel matrix derivative (EMD), mineral trioxide aggregate (MTA), and calcium enriched mixture (CEM) cement.[5-10]

CEM cement has good sealing ability and sets quickly (<1 hour) in aqueous environments.[11] CEM cement is biocompatible and has great ability to induce hard tissue formation, and also possesses considerable antibacterial activity.[12-14] In several studies, favorable results have been achieved using CEM cement in apexogenesis and pulpotomy of permanent teeth, internal and external root resorption, management of furcation perforation and periapical surgery.[12-16]

Generally, genotoxicity tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds that induce genetic damag, gene mutation, chromosomal breakage, and cellular transformation. These tests have gained widespread acceptance as the important and useful indices to determine the carcinogenicity and biocompatibility of compounds.[17,18] Among the above-mentioned tests, gene mutations are reliably measured in bacterial and prokaryotic cells when they cause a change in the growth requirements of the cell. As a result, the Ames assay is one of the crucial tests, which is used routinely to assess the biocompatibility of different compounds.

The *Salmonella typhimurium* assay is a bacterial test widely used to determine the potency of substances that can produce genetic damage and gene mutations. The

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Ames test utilizes *Salmonella* strains with preexisting mutations that are unable to synthesize the required amino acid, histidine, and therefore cannot grow and form colonies in the medium devoid of histidine. New mutations at the site of these preexisting mutations can restore the genes' function and allow the cells to synthesize histidine. These newly mutated cells can grow in the absence of histidine and form colonies.[18,19] The *Salmonella* mutagenicity test was specifically designed to detect chemically induced mutagenesis.[18]

The aim of this *in vitro* study was to compare mutagenic potential of CEM cement as a new VPT biomaterial with that of formocresol and ferric sulfate as conventional materials using the Ames test.

Materials and Methods

Chemicals

CEM cement (BioniqueDent, Tehran, Iran), formocresol (SSA, Produits Dentaires, Switzerland) and ferric sulfate (Astringedent, Ultradent Products, Inc., USA) were used as received or diluted with ultrapure water before administration. Biological grade dimethyl sulfoxide (DMSO), sodium azide (NaN3), 2-aminoanthracene (2-AA), histidine, biotin, glucose-6 phosphate and NADP were purchased from Merck Group (Darmstadt, Germany). Vogel-Bonner (VB) salt solution and glucose solution nutrient broth were from Difco (Difco Laboratories, Detroit, MI). All solutions and media were prepared using ultrapure water, which was obtained through a Millipore Milli-Q Gradient water purification system.

Preparation of rat liver S9 fraction enzymes

Male Wistar rats (5–6 weeks of age) were obtained from the animal house of Tabriz University of Medical Sciences (Tabriz, Iran). Microsomal enzyme system of rats was induced by three daily doses of phenobarbital (80 mg/kg i.p. given as a 16 mg/mL solution in DMSO), as suggested by Elliot et al. (1992).[20] A week after treatment with phenobarbital, the animals were killed by cervical dislocation and the livers were removed and homogenized. Then, the liver S9 fraction, consisting of both microsomal and cytosolic fractions, was obtained by centrifugation of whole-liver homogenate at 9000 × g for 20 min at 4°C.

Mutagenicity assay

TA100 strain of *Salmonella typhimurium* was used to detect the mutagenicity of CEM cement, formocresol and ferric sulfate in the presence and absence of rat liver S9 fraction. The bacterial strain used in the Ames test carries a mutant gene that prevents it from synthesizing the essential amino acid histidine from the ingredients in standard bacterial culture medium.

In this study, negative and positive controls were used to test the accuracy of the experiments. To this end, the negative control was DMSO (1%) and water, while the positive controls, used to compare the results, were strong mutagenic compounds, namely sodium azide (0.5 µg/plate) and 2-aminoanthracene (2.5 µg/plate). The mutagenicity assay was performed according to Ames et al.[21] Briefly, 5 mL of nutrient broth was inoculated by a single colony of *Salmonella typhimurium* (TA100 strain), and then the culture was incubated at 37°C overnight while shaking at 150 rpm. Two mL of melted top agar consisting of agar, sodium chloride and histidine/biotin solution (0.05 mM) was added 50 µL of the overnight culture, as well as different test and control compounds at varying concentrations. Then the mixture was poured on glucose minimal (GM) agar plates. GM agar plate comprises agar, Vogel-Bonner (VB) salt solution and glucose solution (10% v/v). VB salt medium E was composed of warm distilled water, magnesium sulfate, citric acid monohydrate, potassium phosphate dibasic anhydrous, and sodium ammonium phosphate. After solidification of the top agar, the plates were incubated at 37°C for 48 and 72 hours. Following the growth of bacteria on GM agar plates, the histidine revertant colonies were counted manually. Rat liver enzymes (S9 fraction) were used as metabolic activator. To study the effect of enzymatic system on mutagenicity of test compounds, the suspension of *Salmonella typhimurium* in top agar was added the mixture of rat liver enzymes (S9 fraction) and different cofactors such as glucose 6-phosphate and NADP. Subsequently, the plates were incubated for 48 and 72 hours. The number of colonies per plate was then counted and the ratio of the number of histidine revertant colonies to the number of spontaneous revertant colonies for the negative controls was obtained. If the ratio was equal to or greater than 2, the experimental material was considered mutagenic.[19,22]

Results

Figure 1 shows the image of colonies grown on plates with CEM cement (1 in 1000 dilution), ferric sulfate (1 in 1000 dilution), and negative control (1% DMSO), as the representative test results. The numbers of colonies grown on the plates (revertant bacterial colony counts), as well as the mutagenicity ratios for three different pulpotomy agents under different treatments are presented in Table 1. The positive and negative controls used in this study responded as expected. Based on the results presented in Table 1, the mutagenicity ratios obtained for different concentrations of CEM cement in the presence and absence of S9 rat liver fraction are smaller than 2, indicating that at the range of studied concentrations, CEM cement was not mutagen. However, toward the upper limit of the concentration range (i. e., 50 µL of the original solution of CEM cement), it prevented bacterial growth when S9 fraction was added to the culture. In the absence of S9 fraction, the number of bacterial colonies grown on the plates in all concentrations of CEM cement were higher than that shown for the plates containing S9 fraction.

Addition of 50 µL of 1, 10, and 100 times diluted solution of formocresol to the culture medium completely prevented bacterial growth leading to zero colony count on the corresponding plates. At the higher

dilutions (lower concentrations), bacterial growth was observable, particularly in the absence of S9 rat liver fraction, so that the colony counts (196±3) were higher than the average number of colonies for the negative controls (168±29).

The number of colonies grown on the plates to which ferric sulfate was added at different concentrations was higher relative to two other pulpotomy materials, i.e., CEM cement and formocresol. This was particularly more evident when the rat liver S9 fraction was not added to the culture medium. At two concentrations corresponding to one hundredth and one thousandth dilutions of ferric sulfate preparations and in the absence of S9 fraction, the mutagenicity ratios calculated based on the revertant bacterial colony counts were 2.8 and 2.2, respectively. Collectively, under all the experimental conditions except the latter two cases, the Ames test performed with and without the S9 fraction on TA100 failed to detect significant reverse mutations for the studied materials.

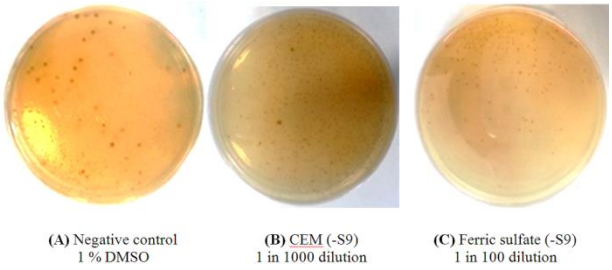

Figure 1. The image of bacterial colonies grown on plates to which were added, **(A)** 50 µL of 1% DMSO (Negative control), **(B)** 50 µL of CEM cement solution (1 in 1000 dilution), and **(C)** 50 µL of ferric sulfate solution (1 in 100 dilution). The negative sign in front of S9 indicates the absence of enzymatic system of rat liver microsomal and cytosolic fractions.

Table 1. The results of Ames test expressed as mean revertant colony counts after exposure to different concentrations of the tested pulpotomy materials in the presence and the absence of metabolic activation induced by hepatic enzymes of rat liver S9 fraction.

Test materials	Dilution factor[c]	Colony counts[a]				Average Ratios[d]			
		TA100[b]							
		+S9		-S9					
		Average	±SD	Average	±SD	+S9	SD	-S9	SD
CEM Cement	1	0	0	55	5	0.00	0.00	0.33	0.03
	10	11	1	116	7	0.07	0.01	0.69	0.04
	100	28	1	70	5	0.18	0.01	0.41	0.03
	1000	30	5	100	6	0.19	0.03	0.59	0.04
	10000	124	3	254	11	0.79	0.02	1.51	0.06
Formocresol	1	0	0	0	0	0.00	0.00	0.00	0.00
	10	0	0	0	0	0.00	0.00	0.00	0.00
	100	0	0	0	0	0.00	0.00	0.00	0.00
	1000	8	1	40	2	0.05	0.01	0.24	0.01
	10000	17	2	196	3	0.11	0.01	1.16	0.02
Ferric Sulfate	1	30	2	54	3	0.19	0.01	0.32	0.02
	10	95	3	260	3	0.60	0.02	1.55	0.02
	100	120	3	462	3	0.77	0.02	**2.75**[e]	0.02
	1000	245	3	370	2	1.56	0.02	**2.20**[e]	0.01

[a]The colony counts are the average of colony counts from three plates. [b]The experimental results for the test materials added to the bacterial culture at the concentrations indicated by the dilution factors. [c]The dilution factor of the test compound from which 50 **µ**L was added to the bacterial culture. The test materials were used as received or diluted using ultrapure water. [d]Each value is the average of ratios for colony counts for a test material at a given concentration to that of the negative control. The average colony counts for the negative controls (1% DMSO and "water") in the presence and absence of S9 fraction were 157±23 and 168±29, respectively. The averages for positive controls (sodium azide and 2-aminoanthracene) were 580±145 (+S9) and 596±79 (-S9). [e]The indicated values are significantly greater than 2 with p-value < 0.000. All other average ratio values are statistically smaller than 2 with the p-value of < 0.000.

Discussion

The aim of this study was to evaluate the mutagenic effects induced by three VPT agents using the popular Ames test. This investigation was carried out using the TA100 strain of *Salmonella typhimurium*. This is an available and reliable biochemical technique for

evaluating mutagenic potential of biomaterials.[18,19,22,23] The preliminary toxicity assay should be performed in the absence and presence of metabolic activation system which is to be used in the final mutagenicity assay.

When histidine-dependent bacteria are grown on a glucose-minimal (GM) agar plate containing a trace amount of histidine, only those cells that revert to histidine-independence (*His+*) state are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few cell divisions; in many cases, this growth is crucial for mutagenesis to occur. The *His+* revertants are easily scored as colonies against the slight background growth. The number of spontaneously induced revertant colonies is relatively constant for each strain. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased.[23]

Several statistical methods have been established for analyzing *Salmonella* mutagenicity data, and all have their strong and weak points. A popular approach that has been widely used is to set a minimum-fold increase, usually 2–3 folds, in revertants (over the solvent control) as a cut-off between a mutagenic and non-mutagenic response.[24] In the present study this approach was used for evaluating mutagenic influence of VPT materials.

The results showed that under experimental conditions used in this study, only ferric sulfate can be considered mutagenic at 1 in 100 and 1 in 1000 dilutions when the rat liver S9 fraction was not added to the culture medium. In contrast, when liver fraction was added to the culture, the colony count ratio values were very small, indicating lack of mutagenicity under these conditions, probably due to the metabolic conversion of ferric sulfate to safer products. Ferric sulfate at the lower dilutions (i.e., higher concentrations) did not show mutagenicity effects, but at the same time the number of colonies are low, which may be due to the toxic effect of ferric sulfate at higher doses. Although, the mutagenicity effect was not observed for formocresol, based on the bacterial growth pattern seen in the presence of this compound, it may be regarded as a toxic material judged from very low number of bacterial colonies grown on the plates prepared using different concentrations of formocresol. For many years there has been controversy over the suitability of formocresol as a vital pulpotomy material, with concerns about the safety of its principal component, formaldehyde. Therefore, there is great interest in the effectiveness of alternative materials such as ferric sulfate.[6] Ferric sulfate has favorable clinical and radiographic success rates compared to formocresol; therefore, ferric sulfate is recommended as a suitable replacement for formocresol, with lower toxicity.[6,25] However, considering its mutagenic activity observed in this study, its preference over formocresol may be scrutinized. The results presented in this work indicate the biocompatibility of the CEM cement shown by the absence of mutagenicity and toxicity compared to formocresol and ferric sulfate solution tested by the Ames test. This finding is consistent with randomized clinical trials[9,10,26] regarding suitable biocompatibility and tissue reactions to this new pulpotomy agent.

Conclusion

Based on the findings of this *in vitro* study, CEM cement is a non-mutagenic and biocompatible material for vital pulp therapy in primary and permanent teeth and its use is recommended over formocresol and ferric sulfate.

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Ethical Issues

Not applicable.

Conflict of interest

The authors declare that there is no conflict of interests.

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