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Mutagenicity potential (affect) of new atraumatic restorative treatment (ART) material incorporated with *Azadirachta indica* (Neem) against *Salmonella typhimurium*

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ABSTRACT

Background: The mutagenicity potential of a new atraumatic restorative treatment (ART) material against *Salmonella typhimurium* without metabolic activity using the Ames test (genotoxicity) was carried out.
Methods and materials: The potential mutagenicity of new atraumatic restorative treatment materials (ART-I and ART-II) was analyzed using the Ames test. The materials were eluted in dimethyl sulphoxide, 0.9% NaCl solution and sterilized de-ionized water and the aliquots were used after an incubation period of 24 h at 37 °C. Mutagenic effects of the materials were tested on *Salmonella typhimurium* strains TA 98 and TA 100 using the standard assay, and in absence of S9 fraction from rat liver.
Result: No mutagenic effects were detected for these new ART materials on *S. typhimurium* TA100. The incubated DMSO extract and 0.9% NaCl extract (50 µl/plate) of the ART-I exhibited a weak mutagenic potential on *S. typhimurium* TA 98. In particular, Aqua extract (50 µl/plate) of ART-II, was associated with a weak mutagenic potential on *S. typhimurium* TA98.
Conclusion: Both ART materials (ART–I and II) exhibited weak mutagenic effects on *S. typhimurium* TA98 whereas no mutagenic effect was detected on *S. typhimurium* TA100. ART-II is safer than ART-I.

1. Introduction

Atraumatic restorative treatment (ART) involves removing carious tooth tissue using hand instruments followed by restoration of the cavity with an adhesive filling material.[1] The major advantage of ART is that it requires minimal resources, and can easily be used in a community settings.[2–5]

There are several types of ART materials available, such as-glass ionomer cements.[6] Their biocompatibility was assayed by Ames (mutagenicity screening) with and without metabolic activity.[7–9]

The Ames assay (developed by Bruce Ames) is a bacterial mutation assay used to determine the mutagenic potential of materials/substances.[10] The salmonella mutagenicity test is frequently used due to its rapid screening technique. The Ames test is sensitive because of its ability to induce mutations in DNA, which are indicative of adverse changes at a cellular level.[11,12]

The Ames assay is routinely used to assess the biocompatibility and anti-carcinogenic effects of dental materials and other compounds.[13] Histidine-dependent bacteria are grown on a glucose-minimal (GM) agar plate which contains a trace amount of histidine. Only those cells that revert to histidine-independence (*His+*) state can form colonies. The number of spontaneously induced revertant colonies is relatively constant for each strain. When a mutagen is added to the plate, the number of revertant colonies per plate increases.[12]

The aim of the present study was to determine the mutagenicity of a newly developed ART material incorporated with *Azadirachta indica* (*Neem*), by testing it against salmonella typhimurium strains using the Ames test.

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Table 1
Composition of new ART material.

Composition of ART material	ART-I (%)	ART -II (%)	Action
Zinc Oxide	20	20	Base of the Cement
Aluminum Oxide	70	70	Base of the cement
Hydrogenated rosin	06	06	Bonding Agent
Sodium Fluoride (NaF)	03	03	Fluoride Agent
Neem extract	06	11.5	Antibacterial agent
Eugenol	40	40	Binding Agent
Ethoxybenzoic Acid (EBA)	60	60	Bonding Agent

Table 2
Mutagenicity of DMSO, 0.9% NaOH and sterile water extraction of ART-I and ART-II in Ames *Salmonella* assay in the absence of S9 fraction.

Solvent	dose (µl/plate)	Mean (SD) Revertant colonies (n = 3)			
		TA 98		TA 100	
		ART-I	ART-II	ART-I	ART-II
DMSO	0	23 (3)	23 (3)	98 (3)	98 (4.5)
	50	45 (2.52)[a,b]	1 (0)[b]	53 (1.52)[b]	24 (2.08)[b]
	100	34 (5.69)[a,b]	0 (0)[b]	31 (1)[b]	16 (1)[b]
0.9% NaCl	0	200 (5)	200 (5)	624 (1)	624 (1)
	50	280 (3.05)[a,b]	144 (1)[b]	72 (3)[b]	184 (1)[b]
	100	187 (2.08)[b]	200 (0.58)[b]	67 (1.53)[b]	99 (1.53)[b]
Sterile water	0	211 (1)	211 (1)	540 (4)	540 (4)
	50	207 (2.31)	223 (1.73)[a]	37 (2)[b]	108 (1.53)[b]
	100	160 (6)[b]	163 (14.18)[b]	21 (1.53)[b]	60 (1.63)[b]
Positive Mutagen					
AZ	5 µg/plate	–		594 (77)	
NPD	2.5 µg/plate	408 (132)		–	

SD – standard deviation; AZ-sodium azide; NPD- 4-Nitro-*o*-phenylenediamine.
[a] Weak mutagenicity.
[b] A significant differences were found between the re-verdant colonies of the test groups and the control group ($P < 0.05$).

2. Materials and methods

2.1. Chemicals

Sodium azide (NaN), 4-Nitro-*o*-phenylenediamine (NPD), magnesium sulphate, citric acid monohydrate, potassium phosphate, dibasic (anhydrous), sodium ammonium phosphate, D-biotin, -histidine, HCl, biological grade dimethyl sulphoxide, dextrose, sodium chloride, ampicillin, bacto-agar and nutrient broth were purchased from Himedia, India. All the solution and media were prepared using sterilized de-ionized water in an ion-free and dust free laboratory environment.

2.2. Test materials and sample preparation

The composition of new ART materials i.e. ART-I and ART-II is given in the Table 1. ART materials with different concentrations of *Neem* extract were prepared by mixing with a liquid containing 60% of EBA (ethoxybenzoic acid) and 40% eugenol. After initial setting, each cement sample was crushed into amorphous form by triturating in a ceramic mortar, uniformly. Zero point 1 g (0.1 g m) of powder of both ART materials was mixed with 2 ml of DMSO separately, and then it was incubated at 37 °C for 24 h. Aliquots were used for mutagenicity screening of the materials. For each composition, the minimum and maximum quantity of aliquots in DMSO of 50 µl and 100 µl were taken respectively. Same procedure was repeated using physiologic saline (0.9% NaCl) and Sterilized de-ionized water.

2.3. Mutagenicity screening

Salmonella typhimurium strains TA98 and TA100 were purchased from MTCC. The standard plate test was carried out following the procedures outlined in detail elsewhere.[11,14,16] Mutagenicity testing was performed on aliquots of ART-I and ART-II separately, to facilitate comparison.

Five millilitres (5 ml) of nutrient broth was inoculated with a single colony of *Salmonella typhimurium* (TA100 and TA 98 strains), and then the culture was incubated at 37 °C overnight. Two millilitres (2 ml) of melted top agar consisting of agar, sodium chloride and histidine/biotin solution (0.05 mM), 100 µL of the overnight culture, as well as different test and control compounds at varying concentrations were added in a test tube and shaken well. Then the mixture was poured on glucose minimal (GM) agar plates, Vogel-Bonner (VB) salt solution, and glucose solution (40% W/V). The VB salt medium was composed of warm distilled water, magnesium sulphate, 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 24 and 48 h. Following the growth of bacteria on GM agar plates, the histidine revertant colonies were counted manually.

In this study, negative and positive controls were used for standardisation. The negative controls were DMSO, 0.9% NaCl (physiological saline) and sterilized de-ionized water. . A strong mutagenic compound ie., 4-Nitro-*o*-phenylenediamine (2.5 µg/plate) for TA98 and sodium azide (5 µg/plate) for TA100 were used as positive controls. The procedure to prepare the reagents and media was followed according to previous studies.[11,16]

Experiments without metabolic activator were carried out in triplicate for each test material (ART-I and ART-II) and also duplicating for each Salmonella tester strain (TA100 and TA98) as per standard protocol[(11).]

2.4. Statistical analysis

The accumulated data was analyzed statistically using software SPSS version 20. The differences between the revertant colonies of the test groups and the control groups were tested with one-way ANOVA. Also, comparison of the revertant colonies between solvent, dose and ART materials, with respect to the control groups, were analyzed by Host statistics test.

2.5. Determining the level of mutagenicity

Dosages higher than the mean of the control group were defined as "mutagenic'. Whereas an increase in single dose was defined as "weak mutagenic".

3. Result

Table 2 is the summary of the result of the Ames test (mutagenicity assay) with salmonella typhimurium strains TA98 and TA100 in the absence of a metabolically active microsomal fraction from rat liver (S9 fraction). It was found that all test materials led to varying degrees of mutagenicity on *S. typhimurium* strains TA98 but did not have any mutagenic effect on *S. typhimurium* TA100.

Both100 µl and 50 µl per plate doses of ART-I eluted in DMSO, and 50 µl per plate dose of ART-I extracted in 0.9% NaCl, elicited weak mutagenic effect on *S. typhimurium TA98*. On the other hand 50 µl dose per plate of ART-II extracted in satirized de-ionized water exhibited weak mutagenic effect against *S. typhimurium* TA98. From the comparison stated above, it is observed that ART-II material is non-mutagenic relative to ART-I material.

4. Discussion

Ames test was developed by Professor Brues Ames in 1970, who worked largely in identifying environmental chemicals causing mutations and cancer. His innovative work used mutant strains of *Salmonella*

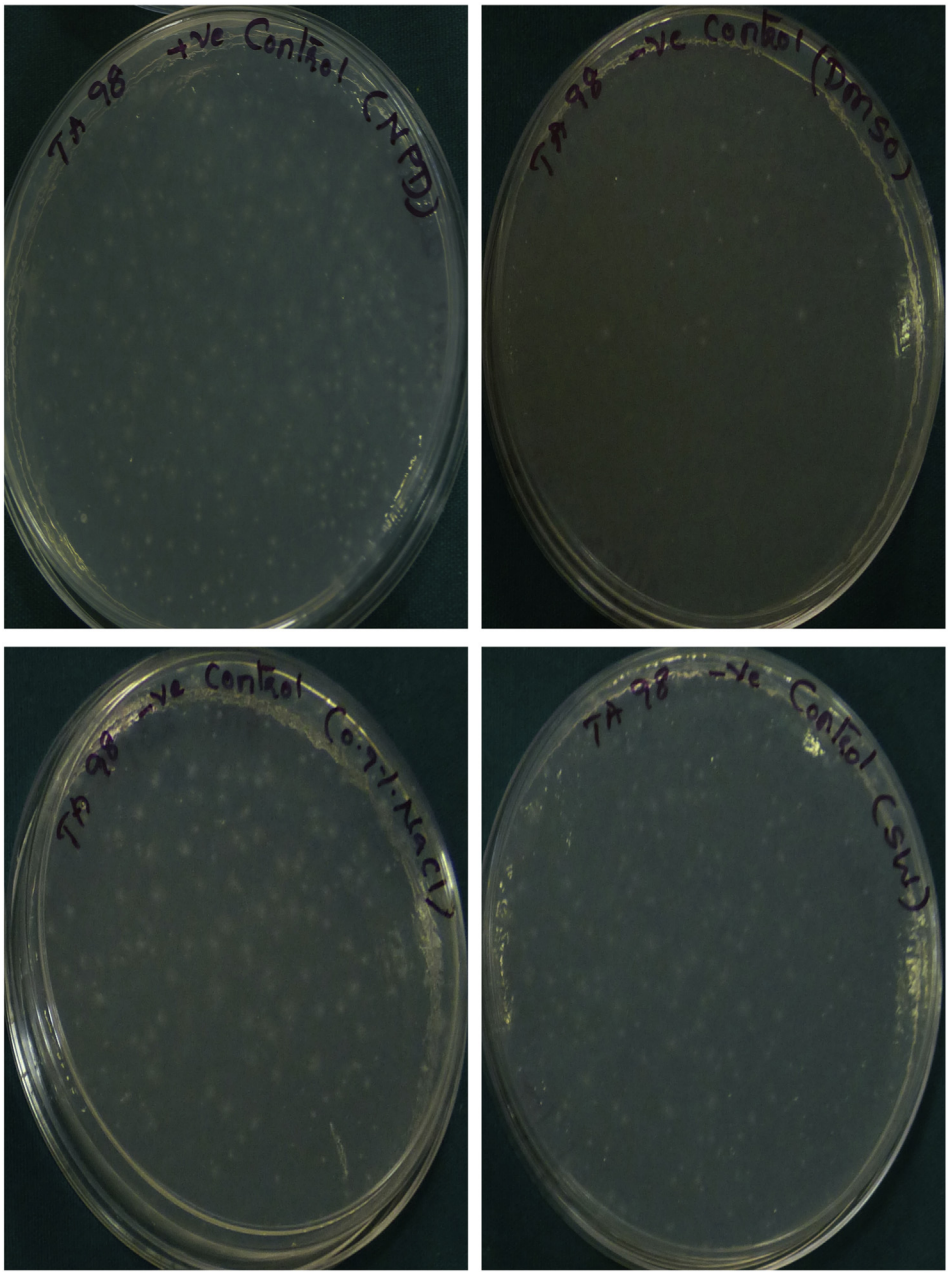

Fig. 1. Positive control: Mutagenic dose response with strain TA98 and 4-Nitro-*o*-phenylenediamine (2.5 μg/plate). Negative Controls: bacterial colonies grown on plates to which were added, 100 μl of DMSO, 100 μl of 0.9% NaCl and 100 μl of sterilized water (SW).

tymphimurium bacteria to identify potential carcinogens without involving mammals.[14]

The Ames assay is used to assess the biocompatibility of dental materials and other compounds. This involves growing Histidine-dependent bacteria on a glucose minimal (GM) agar plate. The number of spontaneously induced revertand colonies is relatively constant for each strain. Addition of a mutagenic compound will increase the number of revertant colonies per plate. It can thus be used to check the mutagenicity of materials, as mutagenic materials should increase the revertant colonies per plate.

New ART material was developed in the laboratory of Fluoride Research Unit of the Department of Oral Biology & Genomic Studies of AB Shetty Dental College, Nitte Deemed to be University, India in collaboration with National Institute of Technology Karnataka, Surathkal and the ratio of composition used was fixed by assessing the physical and mechanical properties such as compressive strength, micro-hardness, solubility properties as per ISO standardised assessment procedure of dental materials[15].

This investigation involved different approaches for the assessment of these new ART materials, which consisted of a basic powder containing zinc oxide, alumina and an acidic liquid of ethoxy benzoic acid and eugenol that are mixed together in a viscous paste.[17] It was then placed in Teflon mould (dimension 7 mm × 3.5 mm) to set into hard mass. This final uniform set of ART material was processed to detect the physical properties by maintaining the similar conditions against a standard.

Two ART materials, ART-I and ART-II were prepared with varying percentage of *Neem* extract. Extraction of *Neem* was by Soxhlet method.[18] Further the physical and mechanical properties, antibacterial property and marginal adaptation to the tooth structure by Scanning Electron microscopy (SEM) for both materials were determined (Study data has not been included in this article).

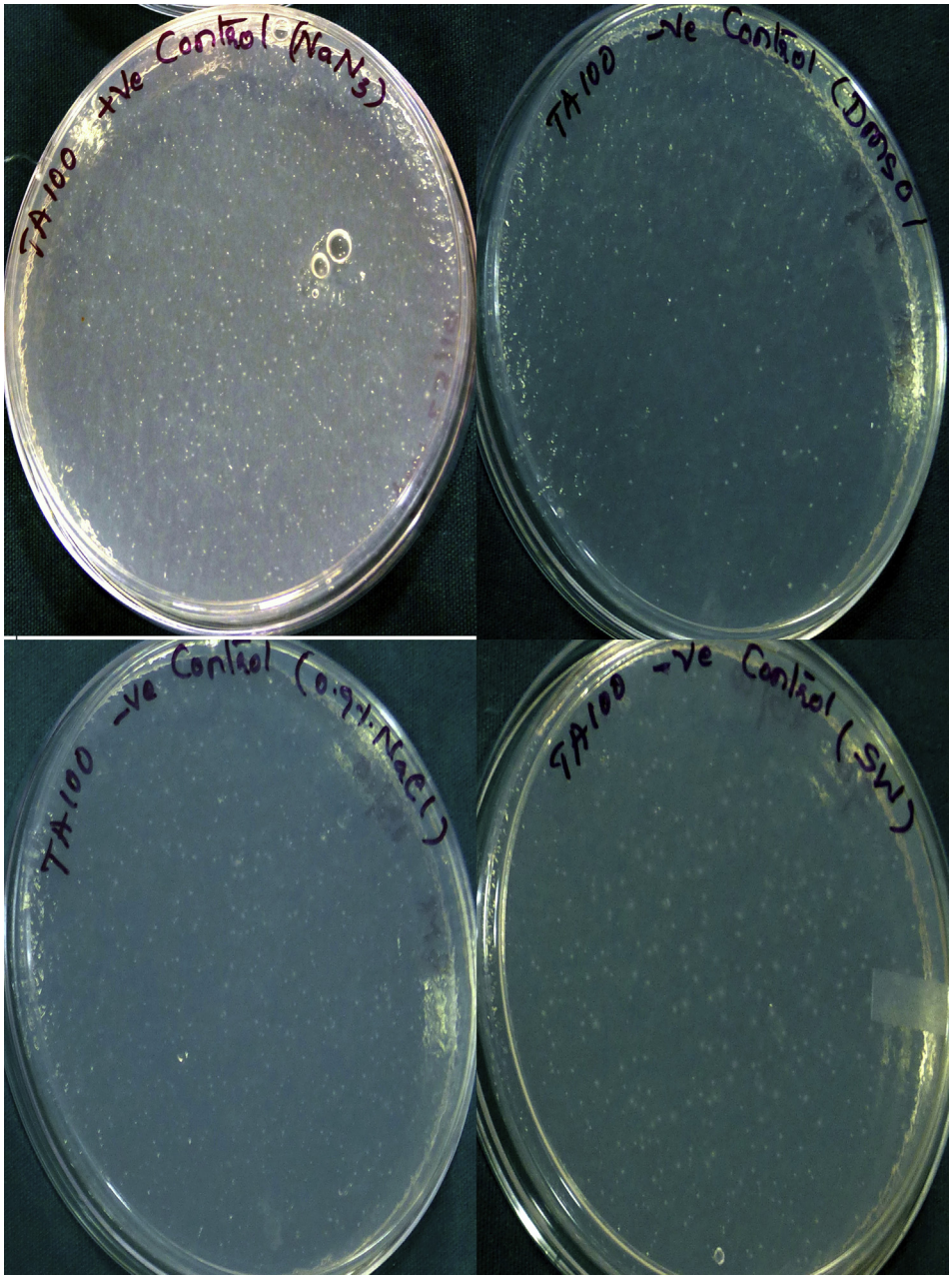

Fig. 2. Positive control: Mutagenic dose response with strain TA100 and sodium azide (5 μg/plate). Negative Controls: bacterial colonies grown on plates to which were added, 100 μl of DMSO, 100 μl of 0.9% NaCl and 100 μl of sterilized water (SW).

In the present study, the mutagenic potential of new ART-I and ART-II materials were explored using the Ames *Salmonella* test without S9 fraction. Formation of colonies in different control groups are shown in Figs. 1 and 2 Both ART materials did not exhibit mutagenic effects on *S. typhimurium* TA100. A similar observation was found in a previous study with a marketed glass ionomer cement, shown to be non-mutagenic against *S. typhimurium* TA100 without the S9 fraction.[19] Significant differences were found between the re-verdant colonies compared between solvents, doses and ART materials ($P < 0.05$).

On the other hand, the incubated DMSO extracts (50 μl/plate and 100 μl/plate) and 0.9% NaCl extract (50 μl/plate) of the ART-I had a weak mutagenic potential on *S. typhimurium* TA 98. Besides, the sterile water extract (50 μl/plate) of the ART-II material showed a weak mutagenic potential on *S. typhimurium* TA98. Statistically there was a significant difference found between the revertant colonies of the different doses of test groups (ART-I and II) extracted from DMSO and 0.9% NaCl and the control groups ($P < 0.05$). But, no significant difference was found between the revertant colonies of the test groups extracted from sterilized water and the control group ($P > 0.05$).

Ames' test is able to detect about 83% of all carcinogens as mutagenic.[19] However, these investigations were conducted without the S9 fraction. Ideally, these tests should include assessment with and without the S9 fraction for evaluation of the mutagenic activity of restorative materials.

The study indicates that ART-II is a relatively safe material for human use. None the less further research based on this initial study needs to be conducted to corroborate these findings.

5. Conclusion

Within the limitations of this study it can be concluded that ART-I and ART-II materials exhibited weak mutagenic effects on *S.*

typhimurium TA98. But, mutagenic effects were not detected for either ART material on *S. typhimurium* TA100. Further research needs to be conducted to corroborate these findings such as, toxicity test in animal and clinical trial for human use as an ART. This may help restore the primary teeth atraumatically, and cost-wise the product would be a less expensive therapeutic restorartive material.

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