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An evaluation of genotoxicity of different root canal sealers by MN assay – An in-vitro study

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Abstract:
Objective: *To evaluate the genotoxicity of Endoflas FS (zinc-oxide eugenol based sealer), RC Seal (epoxy- resin based sealer), Diapex (calcium hydroxide based medicated sealer), Roeko Seal (silicone based sealer) and MTA Fillapex (MTA based sealer) root canal sealers using MN assay.*
Methods: *The samples were divided in to two main groups - positive control group (EMS media alone) and experimental group (sealers used in the study). i. Positive Control group: EMS media alone was used as control group. ii. Experimental group: This group was divided into five subgroups according to the type of root canal sealers used: Group 1: Endoflas FS (EFS), Group 2 :Diapex (D), Group 3 : MTA Fillapex (MF), Group 4 : RC Seal (RCS), Group 5: RoekoSeal(RS). The extracts from the root canal sealers of each experimental subgroup were serially diluted (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32). They were tested for genotoxicity on L929-Mouse fibroblast cells line using MN assay at 24 hours, 48 hours and 78 hours.*
Results: *The average of micronuclei number/1000 cells, formation in EFS was more in 1:1 concentration at 72hr (22.60±0.54 and was less in the concentration 1:32 at 24hr (3.00±0.70). The average micronuclei number/1000 cells, formation were highest in 1:1 concentration at 72hr (21.80±0.44) and 48hr (19.80±0.44),higher than control group and were lowest in the concentration 1:32 at 24hr (2.20±044). The average micronuclei number/1000 cells formation in Group III (MF) was highest in 1:1 concentration at 72 hr (24.20±0.44).there was gradual decrease in micronuclei number/1000 cells in 1:1; 1:2; 1:4 at 24hr, 48hr, 72hr but in 1:8 at 48 and72hr there was no differences micronuclei number /1000 cells formation.*
Conclusion: *It may be concluded that addition of bactericidal agents such as iodoform and paramonochlorophenol in the formula of root canal sealers can augment their genotoxicity.*
Key words: *Genotoxicity, Root Canal Sealers, concentration*

I. Introduction

Eugenol (EUG) is an aromatic compound generally found in cloves, mainly from Syzygium aromaticum (L.) Merr & L. M. Perry. It has a remarkable analgesic effect, which makes it to be used for the treatment of toothaches[1]. Together with zinc oxide, referred to as zinc oxide-eugenol, it is also greatly used in temporary dental fillings[2]. Despite the beneficial effect of this material, the possibility of genotoxic effects cannot be ignored since genotoxicity is one of the side effects of chemical products[3,4].

In the last century, dentistry has acquired great technological advances that sought promptness for the professionals of this area and comfort for the patient. Nevertheless, takinga simple restoration as an example, it is necessary to utilize various chemical elements that, depending on the concentration used, can affect human health and are also capable of causing injury to genetic material[3].

There is a great chance of causing cell mutations, such as: micronuclei, nucleoplasmic bridges, nuclear buds, among others[3]. Micronucleus test, sister chromatid exchange test and genotoxicity analysis are performed to detect the toxicity of elements[5]. The micronucleus test is one of the methods available for the evaluation of spontaneous or induced chromosome damage. Micronuclei are free corpuscles that measure from 1/16 to 1/3 of the size of the nucleus, with a shape from round to oval and are usually found beside the main nucleus, to which they are similar in terms of shape and color[6,7]. The micronucleus originates from a late chromosome region or irregular migrations during anaphase[8].

The aim of this study was to evaluate the genotoxicity of Endoflas FS (zinc-oxide eugenol based sealer), RC Seal (epoxy- resin based sealer), Diapex (calcium hydroxide based medicated sealer), Roeko Seal (silicone based sealer) and MTA Fillapex (MTA based sealer) root canal sealers using MN assay.

II. Material And Methods

The present study was conducted in the Department of Conservative Dentistry and Endodontic, Faculty of Dental Sciences, King George's Medical University, Lucknow in collaboration with CSIR-Indian Institute of

Toxicology Research (formerly, Industrial Toxicology Research Centre), Lucknow. The study was approved by the ethical committee of the institute.

Five endodontic root canal sealers – Endoflas FS (Sanlor,Colombia),Diapex (DiaDent Group International,canada) , RC Seal (Denfills,India),RoekoSeal (Roeko, Langenau, Germany) and MTA Fillapex (Angelus, Londrina, Brazil) were included in this study. The study involved assessment of genotoxicity by means of MN assay.

In the present in-vitro study, samples were divided in to two main groups - positive control group (EMS media alone) and experimental group (sealers used in the study).

1. Positive Control group: EMS media alone was used as control group.
2. Experimental group: This group was divided into five subgroups according to the type of root canal sealers used:
   - Group 1 : Endoflas FS (EFS)
   - Group 2 :Diapex (D)
   - Group 3 : MTA Fillapex (MF)
   - Group 4 : RC Seal (RCS)
   - Group 5: RoekoSeal**(**RS**)**

The extracts from the root canal sealers of each experimental subgroup were serially diluted (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32). They were tested for genotoxicity on L929-Mouse fibroblast cells line using MN assay at 24 hours, 48 hours and 78 hours.

Assessment

a) Cell Line Maintenance:

L929 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) Plus 10% Fetal Bovine Serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were cultured in incubator at 37°C in a humidified air mixed with 5% CO2.

b) Cell line Harvesting/ Sub-culturing of cell:

Cells were taken after 70-80% confluency. Media was decanted and washed with cell culture grade PBS. Cells were trypsinized by using 0.25% trypsin-EDTA solution and leave it for 1 minute. Trypsin was removed completely then media was added and single cell suspension was made by repeated pipetting. Count the cells with trypan blue exclusion dye seed according to $25cm^2$ or $75cm^2$. The normal splitting ratio and cell number doubling time of each cell line are characteristically same for the cell line. Split cell lines according to their normal splitting ratio, that a cell line with 1:2 split ratios, cells grown to complete monolayer in a $25cm^2$ flask could be seeded to two $25cm^2$ flask. Seeding cells in low number not only lengthen the cell harvesting time but at times cell may be under stress due to low/lack of optimal cell-cell contact.

c) Cryopreservation of cell line:

Media of culture flask having 70-80% confluency was decanted; 1 ml trypsin-EDTA solution (on per 5 ml of media) was added to the flask and left for 1 minute; Flask along with trypsin was observed under microscope, rounding of cells due to the detachment from the flask surface could be seen; Trypsin was decanted and left the flask for 1 minute; After 1 minute the flask was tapped hard to detach the cells from the surface of the flask; Adding sufficient amount of fresh media a homogenous single cell suspension was prepared; The cell suspension was taken in a 15 ml tube and centrifuged for 5 minutes; Supernatant discarded, PBS added and centrifuged for 5 min; Supernatant was discarded and 900µl of media and 100ul of 10% Dimethyl Sulfoxide (DMSO) was added to the cell pellet and mixed thoroughly; the cell lysate was transferred to cryovials, date and time of cell line named was marked on cryovials; Cryovials were put into the cryofreezer and kept at 4^0C for 30 minutes. After that the cryovials were transferred to -4^0C and left there for 1hour, after it is transferred to -20^0C and kept for 12 hours and then to -70^0C for a week and for preserving for long time transferred to liquid nitrogen container. Gradual cooling of cells protects cell damage due to cryofreezing effect.

d) Reviving of frozen cells:

Cryopreserved vials or ampules were removed from liquid nitrogen container with a pair of forceps and immediately immersed in 37^0C water bath for rapid thawing. The vials was wiped with alcohol and opened in culture hood. Cells with cryofreezing media were pipetted into 10 ml growth medium in centrifuge tubes and mixed by gentle shaking. Tube was centrifuged for 20 minutes then supernatant was discarded and pellet was mixed with fresh media. If required further washed and centrifuged with fresh media. Finally, pelleted cells were resuspended in fresh media and transferred to culture flasks and incubated at 37^0C with 5% of CO2 incubator. After initial adhering of cells, media can be changed to make sure that cells were completely devoid

of any remaining cryofreezing media. Once cells grown to adequate confluency, split and propagate it into new flask.

e) Preparation of Extract:

The following materials were used: Endoflas FS, Diapex, RC Seal, RoekoSeal and MTA Fillapex. The sealers were mixed according to manufacturer instructions and sealer samples were prepared in 24 well plates (16.2 mm in diameter, 2 mm high), and they were incubated at 37^0 C for 24, 48 and 72 hours immediately after mixing. Specimens were then covered with 2.5 mL cell culture Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, antibiotics-antimycotics solution (100X 1mL per 100 mL) and incubated in dark for 24 h at 37^0 C in a humidified air mixed with 5% CO2. After incubation, these original extracts were then serially diluted (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) in cell culture medium before testing.

Micronucleus assay:

Micronucleus (MN) assay was carried out using standard protocols. Briefly, cells were grown on cover slips placed in 8-well plates in DMEM/F-12 medium. The cells were exposed to different dilutions of extracts of test compounds; cells were incubated up to 43-44 h in fresh medium and blocked for cytokinesis using cytochalasin-B (3 µg/mL). Cells were then harvested by hypotonic buffer (0.075 M KCl) for 5-10 min at 37° C and fixed in Carnoy's fixative (methanol/acetic acid, 3:1). Finally, cells were dropped onto the slides and stained with 5% Giemsa in phosphate buffer (pH 6.8) for 15-20 min and mounted with DPX for microscopic examination. A minimum of 1000 binucleated cells with well-defined cytoplasm in each slide was scored for the presence of MN using a Nikon Eclipse 80i upright microscope attached to a Nikon digital CCD cool camera (Model DS-Ri1 of 12.7 Megapixel). Data presented for MN are the mean of three slides.

Statistical analysis

The results are presented in mean±SD. The one way analysis of variance (ANOVA) was used to compare the means among different concentration at each point of assessment followed by Tukey's post-hoc multiple comparison tests. The p-value<0.05 was considered significant. All the analysis was carried out by using SPSS 16.0 version (Chicago, Inc., USA).

III. Results

The average of micronuclei number/1000 cells, formation in EFS was more in 1:1 concentration at 72hr (22.60±0.54 and was less in the concentration 1:32 at 24hr (3.00±0.70). There was gradual decrease in the of micronuclei number/1000 cells, formations from 1:1 to 1:32 concentration at 24hr, 48hr and 72 hr and was statistically significant ($p<0.001$). The intra-group multiple comparison showed that there was significant ($p<0.001$) difference among all the concentrations at 24, 48 and 72 hour (Table-1).

The average micronuclei number/1000 cells, formation were highest in 1:1 concentration at 72hr (21.80±0.44) and 48hr (19.80±0.44),higher than control group and were lowest in the concentration 1:32 at 24hr (2.20±044). There was gradual decrease in the micronuclei number/1000 cells, formation from 1:1 to 1:32 concentration at 24hr, 48hr and 72 hr and was statistically significant ($p<0.001$). The intragroup multiple comparison showed that there was significant difference ($p<0.001$) among all the concentrations at 24, 48 and 72 hour (Table-2)

The average micronuclei number/1000 cells formation in Group III (MF) was highest in 1:1 concentration at 72 hr (24.20±0.44).there was gradual decrease in micronuclei number/1000 cells in 1:1; 1:2; 1:4 at 24hr, 48hr, 72hr but in 1:8 at 48 and72hr there was no differences micronuclei number /1000 cells formation. The micronuclei number/1000 cells formation decrease with 1:1: 1:2; 1:4; 1:8; 1:16; 1:32 concentration at the 24hr, 48hr, 72hr and was statistically significant ($p<0.001$). The intra-group comparison showed that there was significant ($p<0.001$) difference among all the concentrations at 24, 48 and 72 hour (Table-3).

The average micronuclei number/1000 cells formation in RCS Group was more in 1:1 concentration at 72hr (18.20±0.44). However in 1:4; 1:16 at 24hr,48hr,72hr thre was no difference in micronuclei number/1000 cells formation. There was gradual decrease in micronuclei number/1000 cells formation in 1:1; 1:2; 1:4; 1:8; 1:16; 1:32 concentration at 24hr,48hr and 72 hr and this was statistically significant ($p<0.001$). The intragroup comparison showed that there was significant ($p<0.001$) difference among all the concentrations at 24, 48 and 72 hour (Table-4).

The average micronuclei number /1000 cells formation in RS Group was more in 1:1 concentration at 72hr (22.80±0.44) and in 1:16 concentration at 72hr there was increase in micronuclei number/1000ceels formation. However in 1:32 concentration at 24hr, 48hr, 72hr there was no differences in micronuclei number/1000 cells formation. There was gradual decrease in micronuclei number/1000 cells formation in 1:1; 1:2; 1:4; 1:8; 1:16; 1:32 at 24hr, 48hr, and 72hr respectively and this was statistically significant ($p<0.001$). The intergroup comparison

showed that there was significant ($p<0.001$) difference among all the concentrations at 24, 48 and 72 hour (Table-5).

IV. Discussion

A complete sealing of the root canal system after cleaning and shaping is critical for a successful endodontic treatment. Root canals are traditionally filled with gutta-percha cones and a root canal sealer. Although endodontic sealers are designed to be used only within the root canal during endodontic therapy, sometimes they can extrude through the apical constriction.

Endodontic therapy aims at elimination of residual pulp, tissue breakdown products, microorganisms present inside the root canal system, followed by hermetic filling as possible, perfect apical seal, tissue mineralization induction, immunological compatibility, antimicrobial activity[9]. In addition, Grossman recommended endodontic sealers not to provoke an immune response in periradicular tissue and neither be mutagenic nor carcinogenic.

The present study conducted to evaluate the genotoxicity of Endoflas FS (zinc-oxide eugenol based sealer), RC Seal (epoxy- resin based sealer), Diapex (calcium hydroxide based medicated sealer), Roeko Seal (silicone based sealer) and MTA Fillapex (MTA based sealer) root canal sealers using MN assay.

Genotoxicity can be measured by various methods like Bacterial Reverse Gene Mutation Assay, Sister Chromatid Exchange Teat (SCE), Mouse Lymphoma Gene Mutation Assay, Micronucleus assay (MN Assay), Chromosome Aberration Test and Comet Assay (DPX) measures genotoxicity**.** The Micronucleus assay (MN assay) used in the study is a well-established assay in genotoxicity testing and a frequently used method in human biomonitoring[10].

The study was performed *in vitro.* In vitro techniques include utilizing cell or tissue cultures, isolated cells, tissue slices, subcellular fractions, transgenic cell cultures, and cells from transgenic organisms. Continuous cell lines, like 3T3 or L929 mouse fibroblast are being routinely used for testing of toxicity properties of dental materials because of their reproducible growth rates and biological responses[11]**.**

In the present study, EMS (Ethyl Methanesulphonate) added to the L929-mouse fibroblast cell line cultured in the DMEM media, comprised the positive control group. The EMS was used as positive control in the MN assay because it is proven material that causes large induction of micronuclei formation in L-929 cells, denoting their ability to cause damage to cellular DNA and therefore being considered a highly genotoxic[12].

In the experimental group, L929-mouse fibroblast cell line used for MN assay were treated with the different root canal sealer extracts were prepared aseptically under laminar flow hood. DMEM (phenol free) was used for preparing the root canal sealer's extracts. There are several studies reported in the literature[13,14] where the genotoxicity of various dental materials was measured using extracts (made from the respective material) i.e, by indirect contact.

The MN assay were performed with standard protocols and the cytotoxicity and genotoxicity of different root canal sealer extracts were evaluated at 24hr, 48hr and 72hr time intervals with serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32) on L929-mouse fibroblast cells line.

The genotoxicity of five root canal sealers tested in the present study showed significant differences between dilutions. Our results indicate that immediately after mixing all of the sealers were toxic, although duration and magnitude of their toxicity were different. Most of sealers exert some toxic effect when they are fresh or in short testing times[15-17] .

In the present study, Endoflas FS showed highest micronuclei number/1000 cells formation in 1:1concentration at 72hr compared to other experimental sealer so it can be considered a potential genotoxic root canal filling material and eugenol and paramonochlorophenol, an ingredients of Endoflas FS, were causative agent of genotoxicity. .

The genotoxic effects observed in this study might be related to the release of resinous compounds present in the cement composition as salicylate. This component has stimulated the process of apoptosis in human fibrosarcoma cells and has caused the fragmentation of cell genetic material, determining its precipitation in the cytoplasm[9].

In the present study, RC Seal showed no significant micronuclei number/1000 cells formation. It was speculated that 'long-term' genotoxic action was due to a derivative of bisphenol-A-diglycidyl-ether. Contradictory data have been reported about the mutagenic potential of epoxy resin based sealer in the unset and set condition. A comprehensive screening using four in vitro and in vivo assays (umu, Ames, DIT, AFE) yielded no indication that epoxy resin based sealer may cause mutagenicity in the set condition[18]. However, using the Ames test, a weak mutagenic activity was found in the unset condition and up to 1 day after mixing[19].

In this study, no significant genotoxicity when compared to the positive control. This increase may have been attributed to an error in experiment procedure or experimental method.

No similar published data is available about in vitro genotoxicity of Endoflas FS, Diapex, RC Seal, MTA Fillapex, RoekoSeal root canal sealers. Therefore, validation and extension of our results await further investigations. The results of in- vitro assays may not be directly comparable with the in vivo conditions, where

all healing parameters are functioning. However, the results obtained from in vitro genotoxicity studies could give a general view about the biological effects of dental materials, which can be useful for elucidation of the in vivo conditions.

V. Conclusion

It may be concluded that addition of bactericidal agents such as iodoform and paramonochlorophenol in the formula of root canal sealers can augment their genotoxicity.

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Conflict of interest
None

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Table-1: Comparison of genotoxicity (micronuclei number/1000 cells) within Group I exposed to extracts of Endoflas FS root canal sealer evaluated at 24hr, 48hr and 72 hr and serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) using MN assay

Concentration	N	Mean±SD		
		At 24 hour	At 48 hour	At 72 hour
1:1	5	11.60±0.54	18.60±0.54	22.60±0.54
1:2	5	8.80±0.44	14.60±0.54	14.80±0.44
1:4	5	7.40±0.54	7.20±0.44	8.40±0.54
1:8	5	5.00±0.70	5.20±0.83	5.40±0.89
1:16	5	5.00±0.70	5.20±0.83	5.00±0.70
1:32	5	3.00±0.70	3.20±0.44	3.40±0.54
ANOVA p-value		0.0001	0.0001	0.0001

Table-2: The comparison of genotoxicity (micronuclei number/1000 cells) within Group II exposed to extracts of Diapex root canal sealer evaluated at 24hr, 48hr and 72 hr and serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) using MN assay

Concentration	N	Mean±SD		
		At 24 hour	At 48 hour	At 72 hour
1:1	5	11.00±0.70	19.80±0.44	21.80±0.44
1:2	5	9.40±0.54	13.80±0.44	14.80±0.44
1:4	5	6.00±0.70	8.80±0.44	8.60±0.54
1:8	5	4.40±0.54	4.40±0.54	4.80±0.44
1:16	5	4.00±0.77	3.60±0.89	3.60±0.89
1:32	5	2.20±0.44	3.40±0.54	3.40±0.54
ANOVA p-value		0.0001	0.0001	0.0001

Table-3: The comparison of genotoxicity (micronuclei number/1000 cells) within Group III exposed to extracts of MTA Fillapex root canal sealer evaluated at 24hr, 48hr and 72 hr and serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) using MN assay

Concentration	N	Mean±SD		
		At 24 hour	At 48 hour	At 72 hour
1:1	5	12.40±0.54	18.80±0.44	24.20±0.44
1:2	5	8.80±0.44	8.60±0.54	14.60±0.54
1:4	5	5.80±0.44	6.20±0.44	6.60±0.54
1:8	5	4.40±0.54	4.80±0.44	4.80±0.44
1:16	5	3.40±0.54	3.60±0.54	4.80±0.44
1:32	5	2.20±0.44	2.20±0.44	2.80±0.44
ANOVA p-value		0.0001	0.0001	0.0001

Table-4: The comparison of genotoxicity (micronuclei number/1000 cells) within Group IV exposed to extracts of RC Seal root canal sealer evaluated at 24hr, 48hr and 72 hr and serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) using MN assay

Concentration	N	Mean±SD		
		At 24 hour	At 48 hour	At 72 hour
1:1	5	10.20±0.44	13.00±0.70	18.20±0.44
1:2	5	4.80±0.44	4.80±0.44	5.80±0.44
1:4	5	3.20±0.44	3.20±0.44	3.20±0.44
1:8	5	2.20±0.44	2.80±0.44	2.80±0.44
1:16	5	2.20±0.44	2.20±0.44	2.20±0.44
1:32	5	2.20±0.44	2.40±0.54	2.20±0.44
ANOVA p-value		0.0001	0.0001	0.0001

Table-5: The comparison of genotoxicity genotoxicity (micronuclei number/1000 cells) **within Group V exposed to extracts of Roekoseal root canal sealer evaluated at 24hr, 48hr and 72 hr and serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) using MN assay**

Concentration	N	Mean±SD		
		At 24 hour	At 48 hour	At 72 hour
1:1	*5*	12.20±0.44	18.80±0.44	22.80±0.44
1:2	*5*	8.80±0.44	12.20±0.44	14.80±0.44
1:4	*5*	5.80±0.44	5.80±0.44	8.80±0.44
1:8	*5*	3.20±0.44	5.20±0.44	5.80±0.44
1:16	*5*	2.20±0.44	3.20±0.44	2.20±0.44
1:32	*5*	3.20±0.44	3.20±0.44	4.20±0.44
ANOVA p-value		0.0001	0.0001	0.0001