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A Comparative Evaluation of Genotoxic and Cytotoxic Damage on Oral Mucous Cells of Patients Having Dental Restorations Using Buccal Cytome Assay-An *In Vivo* Study

[*1]Dr. Maria Thomas, [2]Dr. Liza George, [3]Dr. Josey Mathew, [4]Dr. Deepu George Mathew and [5]Dr. Priya Thomas

[*1]Senior Lecturer, Department of Conservative Dentistry and Endodontics, Indira Gandhi Institute of Dental Sciences, Kothamangalam, Kerala, India.

[2]Professor, Department of Conservative Dentistry and Endodontics, Annoor Dental College and Hospital, Muvattupuzha, Kerala, India.

[3]Professor and Head of the Department of Conservative Dentistry and Endodontics, Annoor Dental College and Hospital, Muvattupuzha, Kerala, India.

[4]Professor and Head of the Department of Oral Pathology and Microbiology, Annoor Dental College and Hospital, Muvattupuzha, Kerala, India.

[5]Reader, Department of Oral Pathology and Microbiology, Annoor Dental College and Hospital, Muvattupuzha, Kerala, India

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***Corresponding Author**
Dr. Maria Thomas
Senior Lecturer, Department of Conservative Dentistry and Endodontics, Indira Gandhi Institute of Dental Sciences, Kothamangalam, Kerala, India.

Abstract
Dental caries is the most prevalent chronic disease in both children and adults. Treatment of caries involves restorations which are mostly using amalgam or resin composite materials. Restorative compounds may get released into oral cavity which have various biological effects that needs to be evaluated. Aim of this study is to evaluate and compare the genotoxic and cytotoxic damage on oral mucous cells of patients having different dental restorations and those without any restorations. 30 patients with prior restorations done forms the study group and 30 patients with no restorations done till date forms the control in the study. Exfoliated buccal smears will be obtained by scraping the buccal mucosa with a flat wooden spatula. Smears will then be fixed and stained with Modified Feulgen and Rossenbeck staining technique. For each patient, 500 intact exfoliated cells were examined under digital microscope for the presence of micronuclei and other nuclear anomalies. Krusal-Wallis test was used for statistical analysis. The results obtained showed that the micronuclei frequency in oral mucosa cells had no significant difference in patients with restorations and in restoration-free patients while cytotoxicity biomarkers were present in significantly higher frequencies in patients with restorations than in restoration-free patients. The study concluded that there is no evidence of genotoxic damage induced by restorative materials but cytotoxicity induced by the materials is significant and their long-term effects needs to be evaluated.

Keywords: Genotoxicity, Cytotoxicity, Amalgam, Composite, Micronuclei

Introduction

Oral mucous cells are exposed to components leached from dental restorative materials and their permeability allows penetration of these components released from restorations, resulting in adverse biological reactions [1]. Evaluation of the biocompatibility of restorative materials is the principal requisite for their successful and safe clinical use.

The monomers in resin-based composites are released in the oral environment initially during setting reaction and later due to degradation of the material from the restoration [2]. Polymerization reaction of dental composite is always incomplete and usually leaves a considerable fraction of free monomers, which in turn are released into the oral cavity [3]. Monomers present in the organic matrix also undergo degradation through a variety of mechanisms in the oral cavity which include dissolution in saliva, wear from mastication, interactions with food and drugs, and bacterial activity [4]. The unbound monomers are initially eluted within

the first hours after polymerization and later leachable components are released due to degradation over time [3]. The cytotoxicity of monomers could be because of its ability to interact with the lipid bilayer of cell membranes and cause lipid peroxidation leading to cell death [5]. Bisphenol A (BPA) derivatives like Bis-GMA, Bis-EMA, Bis-DMA resins used in the organic matrix of composites have been demonstrated to act as an endocrine disrupting chemical (EDC) with estrogen like effects causing potential estrogenicity [6].

Dental amalgam which contains mercury as its component is known to cause clastogenic effects. It was suggested that inorganic mercury acts mainly on the cytoskeletal proteins such as kinesin or tubulin, which seems to play an important role in chromosomal segregation thereby resulting genotoxic damage [7]. The cytotoxicity of amalgam aged for 24 hrs appeared to be caused by the combined effect of Hg, Ag, and Cu, and not solely by the unreacted mercury [8]. The constituents from dental amalgam were demonstrated to be released locally and spread systemically and cause toxic and genotoxic alterations [9].

The evaluation of genotoxicity induced by restorative materials is necessary, as there are evidences of relationship between genetic damage and carcinogenesis [10]. Micronucleus is an accepted genomic biomarker and staining with DNA specific stains will help determine the genotoxic damage induced by restorative materials [11]. Cytotoxicity screening assays provide a measure of cell death caused by the materials or their extracts [12]. The nuclear alterations indicating cytotoxic damage include karyolysis, karyorrhexis, pyknosis, binucleated cells, vacuolated cells, nuclear bud and condensed chromatin. Genotoxic and cytotoxic effects of restorative materials on oral mucous cells can be evaluated *in vivo* by the presence of micronuclei and by evaluating the cells in early and late stages of apoptosis [13].

The aim of this study was to evaluate the genotoxic and cytotoxic damage on oral mucous cells of patients having different dental restorations and compare with patients having intact teeth.

Materials and Methods

This in-vivo study was done in the Department of Conservative Dentistry and Endodontics, Annoor Dental College, Muvattupuzha, Kerala, India in collaboration with Department of Oral Pathology and Microbiology, Annoor Dental College, Muvattupuzha, Kerala, India. The study protocol has been approved by the Institutional Human Ethical Committee (IHEC/019-B/41). Sixty healthy patients in the age group of 18-30 years were randomly selected for the study and control group from patients coming to Department of Conservative Dentistry and Endodontics. Patients who are non-smokers and non-alcoholics were included in the study. Patients with prior recent exposures to X-ray and other diagnostic radiation in last 2 months, those with mucocutaneous lesions and allergic diseases and under medication for chronic systemic diseases were excluded from the study. The study group included patients having prior dental restorations done (n=30) and the control group had patients with no dental restorations done till date (n=30).

Patients Were Grouped As

Group A-Patients with no restorations
Group Ba-Patients with prior amalgam restorations
Group Bc-Patients with prior composite restorations

A detailed patient case history was taken specifying patient's demographic details on a patient's proforma. Patient's brushing habits, diet, age of restoration etc were noted. An informed consent was obtained from patients in regional languages.

Sample Collection: Exfoliated buccal smears were obtained by scraping the buccal mucosa with a flat wooden spatula from the patients. Smears were then fixed in isopropyl alcohol and subsequently stained with Modified Feulgen and Rossenbeck staining technique.

Scoring for Nuclear Anomalies Using Image Analysis: Feulgen-stained smears were analyzed using digital microscope at 40 X magnification and images were captured. The slides were analyzed in a zigzag method, capturing series of images.Scoring of nuclear anomalies was done using a semi-automated procedure, with the application of the image analysis software after accurate calibration. The cell counting tool of the software was used and frequencies of micronuclei and metanucleated cells were tabulated and saved in Microsoft excel for further statistical analysis.

For each individual, 500 intact exfoliated epithelial cells were examined for the presence of micronuclei [Figure 1] and other nuclear anomalies- karyolysis [Figure 2], karyorrhexis [Figure 3], nuclear bud and binucleated cells.

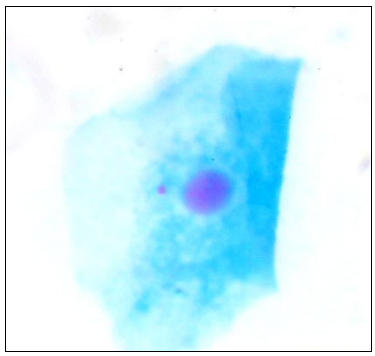

Fig 1: Cell with Micronucleus

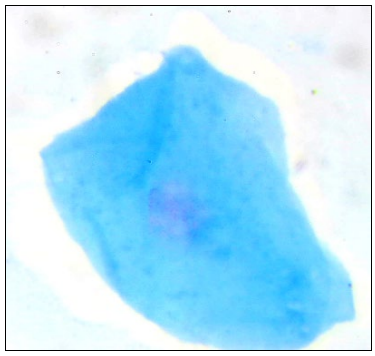

Fig 2: Karyolytic Cell

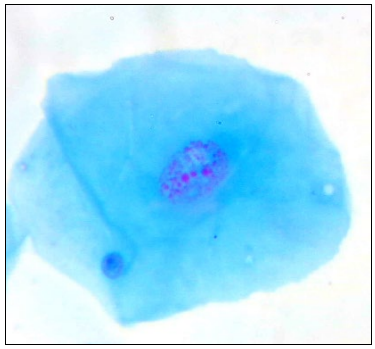

Fig 3: Karyorrhectic Cell

Statistical Analysis: All data were analyzed using SPSS 25.0 software (IBM Corp, US) program. Chi- square test was done to assess the differences between qualitative variable. Quantitative data were tested for normality using Shapiro-Wilks test and non- parametric tests were used. Krusal- Wallis test was performed for comparisons of distribution and median values between the groups. P value was adjusted according to Bonferroni correction. The critical level for statistical significance was set as $p<0.05$.

Results

The mean (±SE) age of the 30 patients having restorations was 22.47±2.066 years and 42.0% of them were males. The age of the 30 patients free of restorations was 23.27 ± 2.149 years, and 48.0% of them were males. The differences based of age and sex were not statistically significant.

The mean number of micronuclei in oral mucosa cells had no significant difference in patients with restorations and in restoration-free patients ($p>0.05$). There was no significant genotoxic damage indicated by micronuclei frequency in any of the patients tested in the study. [Table 1]

Table 1: Distribution of study participants based on the number of micronuclei among the groups

Groups	Mean rank	Mean	P-Value
Control	27.73	0.27±0.450	0.316
Amalgam	34.23	0.53±0.640	
Composite	32.30	0.47±0.640	

* Kruskal Wallis test is applied.

Cytotoxicity biomarkers like karyolysis and karyorrhexis were present in significantly higher frequencies in patients with restorations than in restoration-free patients ($p<0.001$). The mean number of karyolysis and karyorrhexis was significantly higher in patients carrying either amalgam (16.40±1.993 and 19.93±1.981) or composite (16.87±1.642 and 18.60±1.993) restorative material, without any significant difference related to the type of restoration. [Table 2 & 3]

Table 2: Distribution of study participants based on the number of Karyolysis among the groups

Groups	Median	Mean rank	Mean
Control	11	16.00	11.47± 1.525[a]
Amalgam	16	43.70	16.40± 1.993[b]
Composite	17	46.30	16.87± 1.642[b]

* Kruskal Wallis test is applied.
Pairs of [a-b] shows statistically significant difference at $p<0.05$

Table 3: Distribution of study participants based on the number of Karyorhexis among the groups

Groups	Median	Mean rank	Mean
Control	11	15.5	11.17± 1.487[a]
Amalgam	20	48.2	19.93± 1.981[b]
Composite	19	42.8	18.60± 1.993[b]

* Kruskal Wallis test is applied.
Pairs of [a-b] shows statistically significant difference at $p<0.05$

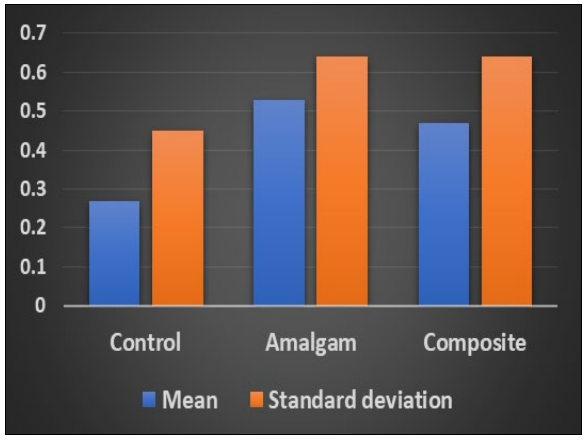

Fig 1: Distribution of study participants based on the number of micronuclei among the groups

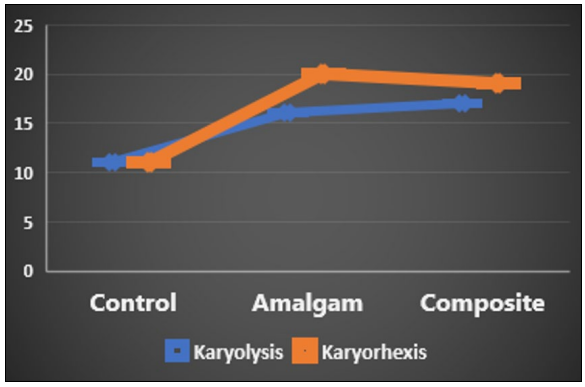

Fig 2: Distribution of study participants based on the number of Karyolysis and Karyorhexis among the groups

Discussion

Degradation of dental restorations occur in the oral cavity under the influence of saliva, food components, and beverages. Clinical degradation is a complex process involving mechanical, chemical and thermal changes and bacterial activity [14]. Oral mucous cells are permanently exposed to the leached components from dental restorative materials and allows penetration of these components. The buccal cytome assay was used in this study to measure DNA damage and is one of the most established and non-invasive procedures [15].

Factors like chewing forces, contact area, type of food intake, degree of polymerization, efficiency of polishing process, pH of saliva, constant temperature changes can influence the composite restorations and lead to breakdown of material [16]. Resin composites may release unpolymerized monomers, additives and filler components in the oral environment [14]. Conditions that affect the amalgam restorations include the biochemistry of the environment, formation of biofilms on the amalgam surfaces, existence of localized corrosion cells, galvanic contacts with other metallic restorations, abrasion during mastication, and synergistic effects of the different forces [17]. Amalgam restorations release metal ions, amalgam debris, non-metallic corrosion products, and mercury vapor into the oral cavity [18].

The study showed that no significant genotoxicity was induced by restorative materials on oral mucous cells. This is in accordance with an in-vivo study done by Tadin A *et al*. in which they evaluated genotoxicity of dental composite materials and concluded that there was no quantifiable evidence of genotoxicity induced by composite restorative materials on buccal mucous cells [19]. The results were in contrast to a study done by Mary SJ *et al*. where they evaluated the genotoxic effects of silver amalgam and composite restorations in oral mucous cells and concluded that both the restorative materials caused genotoxic effects and was significantly higher in patients with multiple restorations [20].

The main mechanism underlying the genotoxicity of dental restorative materials may be ascribed to the ability of released components to trigger the generation of cellular reactive oxygen species and cause oxidative DNA lesions [21]. An in-vivo study by Tadin A. *et al* have shown that, in about six month's period after the restoration, number of micronuclei relapsed to the basal level, indicating a lack of long-term effect on genome stability [22]. So, it was concluded that the observed increase in DNA damage is of temporary nature and may have no biological relevance.

The study results showed that both amalgam and composite restorations induced cytotoxic damage demonstrated by the frequency of cytotoxic biomarkers which reflect consequences of cell injury, cell death and mitotic errors. Patients carrying either amalgam or composite restoration showed almost similar levels of cytotoxicity with no significant difference between them. There are several cytotoxic studies with varying results with respect to amalgam and composite restorations. A study by Ahmed RH *et al*. on human labial and buccal epithelium for cytotoxic effects of fillings concluded that cytotoxicity of amalgam fillings decreased with aging time while that of composite was increased [23]. In an in-vivo study by Mary SJ *et al*., it was stated that composite restorations were least cytotoxic when compared to amalgam restorations [20].

Cytotoxic biomarkers assessed in this study include karyolysis and karyorrhexis. Karyolysis indicates a necrotic type of death associated with cytotoxicity with swelling of the cell and rupture of the cell membrane therefore is a less desirable form of cell death [24]. Karyorrhexis or fragmentation of nuclei is a form of cell death which occurs by apoptosis and is the main morphologic feature of cells dying by apoptosis [24].

This study has a few limitations that may have influenced the results. The type of composite or amalgam restorative materials used in the tested patients is not known. Different materials will have different rates of released components and this will influence the toxicity effects on oral mucous cells. Also, the time elapsed from placement of restoration in different subjects was not taken into consideration. In the oral cavity, numerous factors like dietary habits, tooth brushing habits, consumption of hot food and drinks, and bruxism behaviour could promote the release of restorative compounds, which were not considered in the study. The influence of confounding factors like age and sex were analysed in the study and smokers and alcoholics were excluded from the study. Standardization is difficult in in-vivo investigations which again provides an advantage of toxicity assessment of restorative materials within its natural environment.

Conclusion

Within the limitations of the study, there is no quantifiable evidence of genotoxic damage induced by restorative materials tested in the study. The cytotoxic effects induced in subjects with amalgam and composite restorations were significant when compared to restoration free subjects but their long-term effects need to be evaluated.

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