Genotoxic Effects of Silver Amalgam and Composite Restorations: Micronuclei-Based Cohort and Case–Control Study in Oral Exfoliated Cells

Abstract

Context: A huge number of people carry dental fillings which contain either mercury-based amalgam and/or the recently introduced methacrylate-based resins. It has been shown that both these materials are known to be leached into the oral cavity and induce genotoxic alterations in the buccal mucosal cells. Because of its low cost and ease of manipulation, dental amalgam is still widely used as a restorative material in developing countries. The health risks associated with the components of this restorative material has always been a matter of concern. The present study was designed to assess the frequency of micronuclei (MN) in oral mucosal cells as it is a promising tool for studying the genotoxic effect of clastogenic agents on them. **Aims:** The aim of this study is to evaluate the genotoxic effects of silver amalgam and composite restorations by measuring the mean number of MN in oral exfoliated cells. **Materials and Methods:** The present study was a prospective cohort study which includes a study group consisting of 110 participants. The study sample was equally divided into 55 participants requiring only amalgam restoration and 55 participants requiring only composite restoration in any permanent molar teeth. The same participants before the restoration formed the control group. Smears were obtained from each patient before and 10 days after restoration and were stained with DNA-specific Feulgen stain. The number of cells containing MN out of 500 cells were counted and recorded. After the evaluation of the slides, the results were compiled and subjected to statistical analysis. **Results:** There was a statistically significant ($P < 0.01$) variation in the mean number of MN after the restoration in both amalgam (5.41 ± 1.25) and composite (2.83 ± 0.85) restorations when compared to before the restoration. However, the mean number of MN in composite restoration was significantly less when compared to amalgam restoration. There was also a statistically significant difference in the mean number of MN in subjects with single restoration when compared with multiple restorations in both amalgam and composite restorations. **Conclusions:** The observations from the present study showed the genotoxic effect of amalgam and composite restorations on the oral cavity. However, composite restorations were least cytotoxic when compared to amalgam restoration. Future research and technical advancements are needed for developing safer materials for use in humans.

Keywords: *Amalgam restoration, Feulgen stain, micronuclei*

S. Jeslin Mary,
K. L. Girish,
T. Isaac Joseph,
Pradeesh Sathyan
Department of Oral Pathology, Sree Mookambika Institute of Dental Sciences, Kanyakumari, Tamil Nadu, India

Introduction

A genotoxin is said to be a chemical or agent which cause damage to the DNA or chromosome. Germline mutations are caused by this type of damage to the germ cell. A somatic mutation result from damage to DNA in a somatic cell, which may lead to malignant transformation.[1] Increased genotoxicity was observed in people exposed to mercury by means of diet, working environment, or through dental restorations. Mercury can bind to sulfhydryl groups. Based on this property, mercury

may be involved in four main processes that cause genotoxicity which, includes interruption in microtubule formation, DNA repair mechanisms, generation of oxidative stress, and free radicals and also through direct interaction with DNA molecules.[2]

Incomplete polymerization reaction leads to release of residual monomers from resin-based restorations which interact with tissues of oral cavity. Monomers like 2-hydroxyethyl methacrylate or triethylene glycol dimethacrylate are said to be cytotoxic. They induce genotoxic effects and cause delay in the cell cycle. Monomers also influence the reaction of cells of the

Address for correspondence:
Dr. K. L. Girish,
Department of Oral Pathology,
Sree Mookambika Institute of
Dental Sciences, Kulasekharam,
Kanyakumari - 629 161,
Tamil Nadu, India.
E-mail: klgowda@yahoo.com

nonspecific immunity; inhibit odontoblast cell functions, or delay the odontogenic differentiation and mineralization processes in pulp-derived cells including stem cells. These events point out that resin monomers inexorably alter regulatory cellular networks through interference with signal transduction pathways.[3]

Various *in vitro* studies imply that both mercury-containing amalgam restorations and resin-based dental materials cause deterioration of the cellular pro and antioxidant redox balance. The ability of restorative dental fillings to cause genotoxicity was assessed using comet assay or the single-cell gel electrophoresis by measuring damage to DNA in peripheral blood lymphocytes. Molecular epidemiologic studies use this method to evaluate the genotoxic potential of the classic amalgams and methacrylate directly in humans.[4] Since the cells of oral mucosa are directly exposed to dental materials rather than circulating lymphocytes, they are the first-pass target of the potential harmful effects of these iatrogenic xenobiotics. The oral mucosa is exposed to a high level of dental restorative materials as a result of the salivary pH, brushing forces, chewing habits, and other factors, such as bacterial corrosion and temperature. Furthermore, the genotoxic effect in epithelial cells can involve a higher risk since their high replication index promotes the carcinogenesis process.[5]

Micronuclei (MN) are considered as fragments or whole chromosomes, which during mitosis did not reach spindle pole and remained encapsulated at telophase in a separate nucleus. The chromosome aberration assay detects only the genome damage, whereas micronucleus assay additionally detects chromosome loss or malfunction of mitotic spindle caused by aneugenic mechanisms.[6] Boller, Schmidt, and Heddle were the first to suggest the term micronucleus test in the early 1970s, who demonstrated that this assay provided a simple method to detect the genotoxic potential of mutagens after *in vivo* exposure of animals using bone marrow erythrocytes.[7]

The buccal cell MN assay is potentially an excellent candidate to serve as a biomarker for genetic damage. A rise in the numbers of MN in exfoliated cells indicates an increased rate of genotoxicity. Moreover, MN assay using Feulgen reaction is more specific to detect DNA damage since the reaction is DNA specific.[8] The purpose of this study was to quantify and to compare the genotoxic effects of silver amalgam to resin-based restorations to get a better understanding of the same in our population using MN assay which is an excellent biomarker for genetic damage.

Materials and Methods

The study sample and the control group were selected from the patients visiting the department of conservative dentistry and endodontics. Patients undergoing amalgam and composite restorations were selected as the study group and the same patients before the restoration were used as the control group. Based on formula derived for sample size calculation, 110 participants were included in the study which consisted of fifty-five participants requiring amalgam restoration in any permanent molar teeth (Group I) and 55 participants requiring composite restoration in any permanent molar teeth (Group II). Participants were selected based on the inclusion and exclusion criteria [Table 1]. All the participants were explained the need and design of the study. Only those participants who gave a signed inform consent on an approved document, participated in the study. The participant's demographic data and general history were entered in a predesigned pro forma. Clinical examination of the oral cavity was carried out in the study group wearing sterile hand gloves and mouth mask under artificial illumination.

Collection of exfoliated cells

Participants were asked to rinse their mouth gently with saline to remove debris. Mucosal cells were scraped from the buccal mucosa adjacent to the tooth to be restored using a slightly moistened wooden spatula. The cells were immediately smeared on clean microscopic slides. Just before drying, the smears were fixed with commercially available alcohol spray fixative [Figures 1 and 2]. The slides were then coded and were fixed in Clark's fixative. After proper isolation of the tooth to be prepared, cavity preparation was done, and restoration of the cavity was done as indicated. The participants were recalled after 10 days and again smears were taken from buccal mucosa adjacent to the restored tooth and fixed. The smears were stained with DNA-specific Feulgen stain.

Interpretation of results

The stained slides were observed using research microscope attached to an operating camera to capture the images. Five hundred cells from each sample were focused under light microscope and the numbers of micronucleated cells were counted [Figure 3]. After the evaluation of the slides, the scoring of MN was done according to the criteria established by Tolbert *et al*.; 1991 [Table 2].[9]

Table 1: Inclusion and exclusion criteria for selection of study sample

Inclusion criteria	Exclusion criteria
Participants requiring silver amalgam filling in any permanent molar tooth	Participants with preexisting silver amalgam/composite restorations
Subjects requiring composite fillings in any permanent molar tooth	Subjects with tobacco habits, pan chewing, and alcohol intake
Both males and females	Participants with systemic diseases
Subjects aged 18-35 years	Subjects exposed to radiation in the past 2 weeks

Statistical analysis

The results obtained were compiled and analyzed using IBM statistical software SPSS version 16. Unpaired *t*-test was applied to find the statistical significance before and after the restoration within the groups. ANOVA (*post hoc*) followed by paired and unpaired *t*-test was applied to find the statistical significance between the groups.
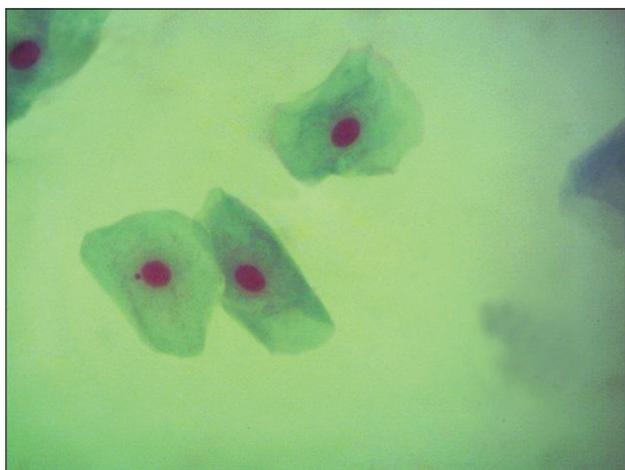
Results

In the present study, the MN frequency was analyzed before and after amalgam and composite restorations. The mean number of MN per 500 cells before the restoration, after the restoration both in Groups I and II with single and multiple restorations were assessed. The results were expressed as mean ± standard error of the mean and were statistically analyzed.

The mean number of MN per 500 cells before and after the restoration in Group I was 1.05 ± 0.75 and 5.41 ± 1.25, respectively, with $P = 0.001$ which was statistically highly significant. Similarly, the mean number of MN per 500 cells before and after the restoration in Group II was 0.96 ± 0.50 and 2.83 ± 0.85, respectively, with $P = 0.001$ which is statistically highly significant. The mean number of MN per 500 cells before and after the restoration in Group I with single restoration was 0.97 ± 0.81 and 4.97 ± 0.88, respectively. The mean number of MN per 500 cells before and after the restoration in Group II with single restoration was 0.93 ± 0.48 and 2.59 ± 0.57, respectively, with $P < 0.05$ which is statistically significant [Tables 3 and 4].

The mean number of MN per 500 cells before and after the restoration in Group I with multiple restorations was 1.25 ± 0.62 and 7.08 ± 1.08, respectively. The mean number of MN per 500 cells before and after the restoration in Group II with multiple restorations was 1.12 ± 0.64 and 4.25 ± 0.88, respectively with $P < 0.05$ which is statistically significant [Table 5].

The mean number of MN per 500 cells before the restoration in Group I and Group II in participants with single restoration was 0.97 ± 0.81 and 0.93 ± 0.48 respectively, with $P > 0.05$ showing no significant difference between the groups before the restoration. The

Figure 1: Armamentarium for smear preparation

Figure 2: Smear taking from the patient

Figure 3: Photomicrograph showing micronuclei (Buccal smear, Feulgen stain, ×100)

Table 2: Tolbert *et al*. criteria for identification of micronuclei

Rounded smooth perimeter suggestive of a membrane

Less than one-third the diameter of the associated nucleus, but large enough to discern shape and color

Staining intensity similar to that of the nucleus

Same focal plane as nucleus

Absence of overlap with, or bridge to, the nucleus

Table 3: Comparison of micronuclei before and after restoration between amalgam and composite

Samples	Number of micronuclei per 500 cells (mean±SEM)		*P*
	Amalgam	**Composite**	
Before restoration	1.05±0.75	0.96±0.50	<0.05
After restoration	5.41±1.25	2.83±0.85	<0.001

SEM: Standard error of mean

mean number of MN per 500 cells after the restoration in Group I and Group II in participants with single restoration was 4.97 ± 0.88 and 2.59 ± 0.57 respectively, with $P < 0.001$ showing significant difference between the groups after the restoration. The mean number of MN per 500 cells before the restoration in Group I and Group II participants with multiple restorations was 1.25 ± 0.62 and 1.12 ± 0.64, respectively, with $P > 0.05$ showing statistically no significant difference between the groups before the restoration. The mean number of MN per 500 cells after the restoration in Group I and Group II in participants with multiple restorations was 7.08 ± 1.08 and 4.25 ± 0.88, respectively, with $P < 0.001$ showing statistically significant difference between the groups after the restoration [Graph 1].

The mean number of MN per 500 cells after the restoration in Group I with single restoration and multiple restorations was 4.97 ± 0.88 and 7.08 ± 1.08, respectively. $P < 0.001$ shows statistically significant difference within the groups with single and multiple restorations. Similarly, the mean number of MN per 500 cells after the restoration in Group II with single restoration and multiple restoration was 2.59 ± 0.57 and 4.25 ± 0.88, respectively, with $P < 0.001$ showing statistically significant difference within the groups with single and multiple restorations [Graph 2].

Discussion

Any substance which is capable of inducing DNA damage and leading to mutation in a cell or cancer is said to be genotoxic. The cells exposed to these genotoxic agents can be assayed for DNA damage by various biomarkers. One such biomarker is the micronucleus assay. The DNA damage can be in any of the forms which include loss of excision repair, single- and double-strand breaks, cross-linking, point mutations, alkali labile sites, structural, and numerical chromosomal aberrations.[10] During the anaphase stage of cell separation, delusion in segregation of chromosome lead to the formation of a lagging chromosome or a chromosome part that become lost and are excluded from the reforming nuclei. These laggards are seen in the cytoplasm as MN.[11]

Exfoliative cytology serves as a method of choice for monitoring populations exposed to genotoxic agents by studying the frequency of MN in the exfoliated cells. The presence of MN within these cells are associated with genetic defects in genome maintenance, exposure to genotoxic agents, accelerated aging, oral cancer risk, and neurodegenerative diseases.[12] MN is considered as small detached portion of extranuclear DNA which is formed during cell division. This serves as a marker of DNA damage and hence used to determine the cellular damage caused by genotoxic agents.[13] The buccal cell micronucleus assay was first proposed in 1983, and it continues to be an excellent biomarker of genetic damage in

Table 4: Comparison of micronuclei before and after restoration between amalgam and composite with single restoration

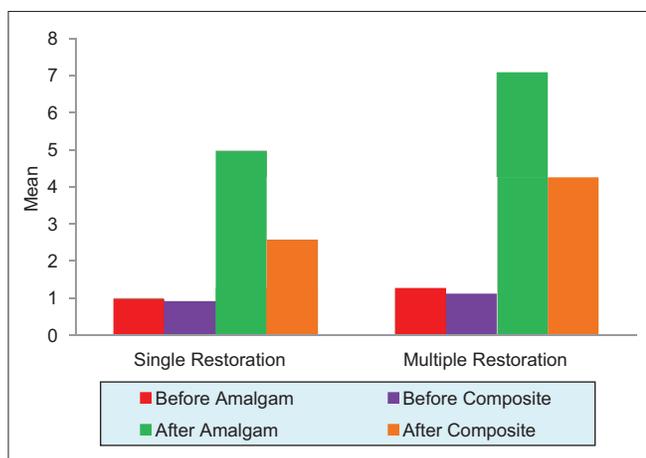| Samples (single restoration)	Number of micronuclei per 500 cells (mean±SEM)		P
	Amalgam	Composite	
Before restoration	0.97±0.81	0.93±0.48	>0.05
After restoration	4.97±0.88	2.59±0.57	<0.001

SEM: Standard error of mean

Table 5: Comparison of micronuclei before and after restoration between amalgam and composite with multiple restoration

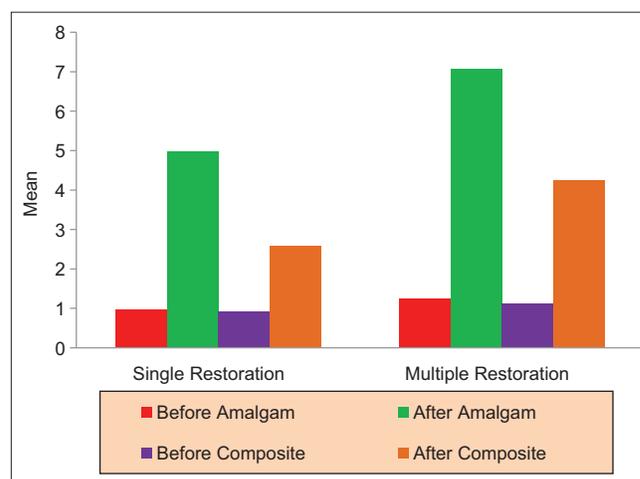| Samples (multiple restorations)	Number of micronuclei per 500 cells (mean±SEM)		P
	Amalgam	Composite	
Before restoration	1.25±0.62	1.12±0.64	>0.05
After restoration	7.08±1.08	4.25±0.88	<0.001

SEM: Standard error of mean

Graph 1: Comparison of mean number of micronuclei before and after the restoration between amalgam and composite with single and multiple restorations

Graph 2: Comparison of mean number of micronuclei before and after the restoration within amalgam and composite with single and multiple restorations

various applications.[11] In oral cavity, MN is considered to be important biomarkers for analyzing the genotoxic effects of various substances with clastogenic and aneugenic modes of action.[14] In this study, the micronucleus assay was applied to verify the genotoxic effects of well-known dental restorative materials, amalgam and composite and to compare both.

The major advantage of exfoliative cytology is the noninvasive character of the technique, which allows a simple and pain-free collection of intact cells from different layers in the epithelium for microscopic examination and quantitative evaluation.[15] Since the cells of oral mucosa are directly exposed to dental materials rather than circulating lymphocytes, they are the first-pass target of the potential harmful effects of these iatrogenic xenobiotics. The oral mucosa is exposed to a high level of dental restorative materials as a result of the salivary pH, brushing forces, chewing habits, and other factors, such as bacterial corrosion and temperature.[5] The oral epithelium has a faster rate of renewal; its turnover time was found to be 7–21 days.[16] The maximal rate of formation of MN is expected at 1–3 weeks after the exposure to a genotoxic agent.[17] In the present study, oral exfoliated cells are taken before and 10 days after the restoration. Waingade and Medikeri in 2012 said that Feulgen stain was used by many researchers because of its DNA specificity. It also facilitates clear identification of MN.[17] In the present study, the DNA-specific Feulgen stain was used.

Dental amalgam which contains mercury is a known clastogen. 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 in the formation of MN. In an *in vitro* study conducted by Thomas *et al*. in 2004, the genotoxic effect of inorganic mercury was studied using the micronucleus assay. They observed the interaction of inorganic mercury with microtubule network of cells leading to the induction of MN.[18]

This clastogenic action of mercury on the cytoskeletal proteins during cell division is the main etiology for the production of MN in the cells. This was supported by the results of the present study where a statistically significant increase in the mean number of MN was observed in the study group of participants with amalgam restoration (5.41 ± 1.25) when compared to control group (1.05 ± 0.75). In a study conducted by Visalli *et al*. in 2012, they evaluated the genotoxic damage in the oral mucosa cells of subjects carrying both amalgam and composite fillings. They observed that the MN frequency in oral mucosa cells was significantly and consistently higher in subjects with restorative fillings than in filling-free subjects. However, in their study, the increase in MN frequency in oral mucosa cells was not affected by the type of restorative material.[5] Both Hg and resin-based

restorative materials were demonstrated to be released locally and spread systemically and cause toxic and genotoxic alterations.

In the present study, there is a statistically significant difference in the mean number of MN in participants with composite restoration in the study group (2.83 ± 0.85) when compared to the control group (0.96 ± 0.50). However, the difference was to a minimal extent. Cytotoxicity of dental composites was steadily associated to the release of residual monomers because of degradation processes or incomplete polymerization of the materials. Studies suggest that uncured resinous materials were considered more cytotoxic than cured materials.[19] Reichl *et al*. in 2006 conducted an *in vitro* study on cytotoxicity of dental composite monomers and the amalgam component Hg2 in human gingival fibroblasts wherein they inferred that the Hg from amalgam is more toxic than resin composite components.[20]

In the present study, the mean number of MN in participants with amalgam restoration (5.41 ± 1.25) was higher and statistically significant when compared to composite restorations (2.83 ± 0.85) which favors the study conducted by Reichl *et al*. Occurrence of genotoxic effects in circulating lymphocytes was demonstrated by a significant dose-effect relationship, with a higher number of dental fillings being correlated with a greater level of DNA damage. In the present study, there was a statistically significant difference between the participants with single restoration when compared with the participants with more than one restoration in both amalgam and composite restoration.

Conclusions

The present study was done to observe and to compare the micronucleus assay among participants with amalgam and composite restorations before and after the restoration. We can conclude from the present study that micronucleus assay in exfoliated cells serves as a specific biomarker of genotoxicity, for studying the genotoxic effects on oral mucosal cells. Our *in vivo* data suggest that restorative dental fillings, to which a huge number of people are continually exposed for long duration, produce DNA damage locally in the oral cavity due to the release of either mercury, or methacrylate. This suggests a greater risk for implication of these materials in human cancer. Hence, more research on a large scale is necessary to study the genotoxic effects of mercury and methacrylate on oral cavity with respect to amalgam and composite restorations, to establish its potential cytotoxic effects. Future research and technical advancements are needed for developing safer materials for use in humans.

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Conflicts of interest

There are no conflicts of interest.

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