Journal of Applied Dental and Medical Sciences
NLM ID: 101671413 ISSN:2454-2288
Volume 3 Issue2 April-June 2017

Original article

Genotoxicity evaluation of Sixth, Seventh and Eighth generation dentin bonding agents by single cell gel electrophoresis technique in human lymphocytes

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A R T I C L E I N F O

Keywords:
Dentin bonding agents, composite resins, Genotoxicity

A B S T R A C T

The main aim of introducing dentin bonding agents is to enhance the bonding of composite resins to dentin as well as minimizes the microleakage at tooth restoration interface. Dentin bonding agents have different chemical compositions, mechanism of action, and clinical application procedures. Therefore different biological effect on the pulpal tissues, could be ranged from severe to none it again depends on several other factors. The use of dental adhesives in routine dental practice has raised questions about their biologic safety. Since, several brands and generations of bonding agents are now available based on clinical use and implication, but their biocompatibility could be a relevant aspect of the clinical success of those materials. **Objectives:** To evaluate the genotoxicity of three different dentin bonding agents Clearfil SE Bond, Gluma self-etch, Futura Bond by comet assay.**Methods**: Genotoxicity evaluation of different dentin bonding agents was carried out in vitro in human lymphocytes at different elution concentrations using the comet assay. Statistical comparison of the results was carried out by ANOVA. **Results**: One of the tested dental adhesives i.e, Clearfil Se bond revealed a statistically significant increase in the tail length, tail intensity or tail moment in treated lymphocytes, independent of the dose related. A slight increase in the tail length and intensity of DNA molecules was observed in Gluma and Futura bond of the elution period at the lowest dilution. **Conclusion:** under the conditions used in this study, all adhesives had acceptable biocompatibility in terms of genotoxicity.

Introduction

Genotoxicity assessment of newer adhesives and primers is essential since these are directly placed on vital dentin in most clinical situations.[1] Bisphenol A glycidyl methacrylate (bis-GMA), triethyelene glycol dimethacrylate (TEGDMA), urethane dimethacrylate (UDMA) and hydroxyethyl methacrylate (HEMA) these are resinous monomers used in restorative dentistry and are formed by different organic molecules that unite as copolymeric chains.[5]

Under clinical conditions if the dental resins composites and resin-based bonding agents undergo incomplete polymerization it may result in free resin monomers of the bonding materials being released from resin matrix into aqueous environment of the oral cavity or into the dentin-pulp complex; Even after polymerization some components can be released.[3,8,11,14.] However, does dentine bonding agents have adverse effects on the health is the question of concern.[4] The monomers can manifest a variety of

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severe health effects such as irritation to skin, eyes, and mucous membranes, allergic dermatitis, stomatitis, asthma, neuropathy, disturbances of CNS, liver toxicity and disturbances in fertility, the same was experimentally and clinically documented, such monomers widely used in dentistry and medicine is Methyl methacrylate(MMA) that also causes abnormalities/Lesions in several organs.[2]

In genotoxicity, comet assays are the most commonly used tests and this technique is very advantageous in the individual cells, for the detection of DNA damage, the comet is the rapid and sensitive method that is induced by a variety of genotoxic agents. Ostling and Johnson introduced this method in the year (1984), and Singh et al. (1988) and Olive(1989) modified this method independently. Tail length, tail fluorescence intensity and tail moment are the three important parameters which are produced during image analysis (Collins et al.,1997). In various in vitro as well as in vivo studies to evaluate DNA damage and repair, comet assay method is used hence, the interest in the comet assay is increasing.[13] In this technique, a very small number of cells are required and the low levels of the DNA are detected. This technique is cheaper and execution is also easy and the results are displayed very quickly. The mechanism underlying the Genotoxic effect or the exact chemical or chemical component causing breaks cannot be identified by this technique.[16]

The studies on genotoxicity of dentin bonding agents are very limited. The experimental data show that resin-based dental materials enhance intracellular reactive oxygen species (ROS), which are well known potential genotoxic element implicated in human chronic degenerative diseases including cancer and cause oxidative DNA damage.[5] In the present study, diploid cells like lymphocytes were preferred to record even minimal effects on the DNA level.

Currently, sixth and seventh generation dentin bonding agents are based on self-etch technique. Due to its advanced clinical tools and devices, nano dentistry has played a major role in the field of restorative dentistry. Nano-sized fillers present in nano-composites and nano adhesives were one of the important components of nano dentistry. Nano particles in the solutions of nano adhesives act to prevent agglomeration which helps in producing high dentine and enamel bond strength, long shelf life, durable marginal seal and release of fluorides. Recently dentine adhesives containing nano fillers have been introduced. One of the manufacturers of dental adhesives (futura bond dc, voco, Germany) have claimed one such system to be the Eighth generation.[9] Dental adhesives are significantly biocompatible, that manufacture a stable relationship with biologic tissues and permit both healing and tissue differentiation. The scientific evidence on adhesives is contradictory. Some authors claimed that they are very safe and might be used even in direct contact with the pulp, whereas others believe that they are not appropriate for direct pulp capping due to reported associated symptoms of persistent inflammation. Some claim that dental adhesive systems contain certain components that may release into the oral cavity and show biological activities (cytotoxicity, carcinogenicity, mutagenicity, genotoxicity) within the body.[15]

Thus, the aim of the study was to evaluate the genotoxicity of Eighth generation dentin bonding agents along with two different dentin adhesive materials.

Materials and methods:

Dentin bonding agents	Manufacturer	Composition
Clearfil SE bond Primer	Kuraray Dental Ltd., Izmir.,Turkey	MDP, HEMA,Hydrophilic aliphatic dimethacrylate, dl-Camphorquinone, N,N-Diethanol-p-toluidine
Clearfil SE bond	Kuraray Dental Ltd.,Izmir.,Turkey	MDP,Bis-GMA,HEMA,Hydrophobic aliphatic dimethacrylate dl-Camphorquinone, N,N-Diethanol-p-toluidine,Colloidal silica
Gluma self etch	Heraeus Kulzer, Hanau, Germany	Glutaraldehyde and HEMA
Futura Bond	Voco,GmbH,Germany	Acid modified methacrylate (methacrylate ester), HEMA, Camphorquinone. Water, Ethanol, Silicium dioxide

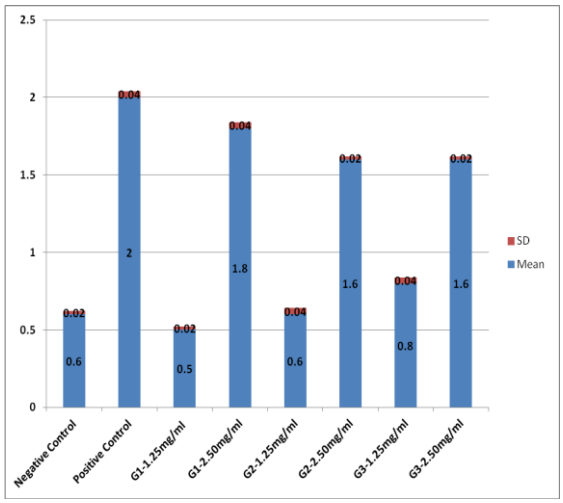
Blood samples:

Fresh blood samples were collected by venepuncture from healthy, non-smoking donors at the Out Patient Department of Conservative dentistry and Endodontic, Navodaya dental college and hospital, Raichur and it was stored adhering to CDC infection protocols.

For each experiment, 5 ml of heparinized (50 unit's mol 1 sodium) whole blood was collected. The donor participated voluntarily and provided oral consent.

Lymphocytes were isolated by Histopaque-1077 and washed with phosphate buffered saline (PBS). PBS is generally utilized to maintain cells for the short term in a viable condition. Cell concentrations were adjusted to approximately $2x10^5$ mL in the buffer. The cells were suspended in a total volume of 1 mL, and each reaction contained 50 lL suspensions (approx.10^4 cells), varying microliter amounts of the test agent (Clearfil SE Bond, Gluma bond and Futura bond) and PBS buffer in a total volume of 1 mL; 1.25 and 2.50mg ml^{-1} concentrations of the dentine bonding agents were examined. As dose or concentration increases, there will be high damage of DNA cells (Kaya et al 2008).These concentrations were chosen according to ISO standard 10993-1. The cells were incubated for 1 h at 37^oc in an incubator together with untreated control samples. Each experiment included a positive control, which was hydrogen peroxide at the concentration of 50 l mol L^{-1} . All test substances were

Taillength



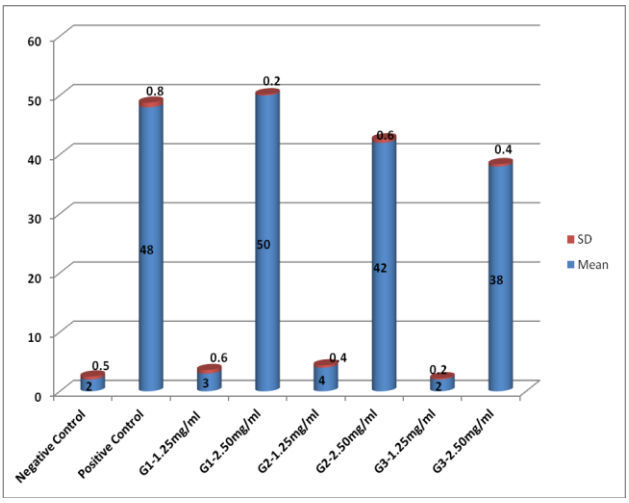
Tail Length of Group1: Clearfil SE bond, Group 2:Gluma bond, Group 3:Futura bond treated human peripheral lymphocytes.

Where as G1:Group 1;G2:Group 2; G3:Group 3

Negative control:PBS(Phosphate Buffer Saline)

Positive control:H_2O_2

Tail intensity:



Tail intensity of Group1: Clearfil SE bond, Group 2:Gluma bond, Group 3:Futura bond treated human peripheral lymphocytes.

Where as G1:Group 1;G2:Group 2; G3:Group 3

dissolved in PBS with a concentration of 50 mg mL^{-1} and incubated for 24 h at 37°c.

After incubation, the lymphocytes were harvested by centrifugation at 800 g for 3 min at 4°c, and the cells were suspended in 75 lL low melting agarose (LMA) for embedding on slides. The replicate experiments were carried out with blood samples from the same donor collected at different time intervals. An aliquot of cells was used to check for viability by trypan blue exclusion.

In this test cell, suspension is simply mixed with the dye (trypan blue) and then visually examined to determine whether cells take up or excludes dye. A viable cell exhibits clear cytoplasm, whereas a non-viable cell will have a blue cytoplasm.

Slide preparation:

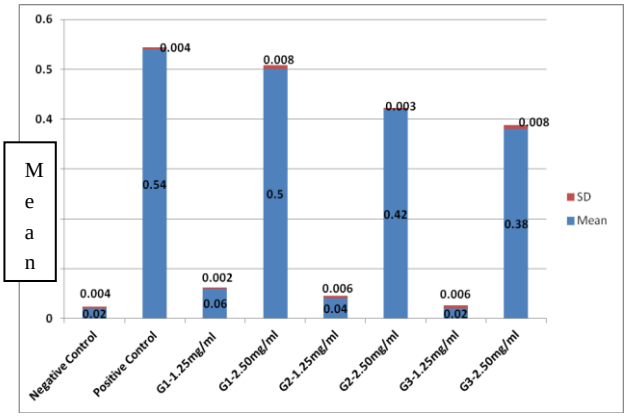
The technique followed was the basic alkaline technique of Singh et al. (1988), as further described by Collins et al. (1997). Microscopic slides were pre-coated with 1% normal melting agarose at about 4 °C in Ca^{2+}- and Mg^{2+}-free PBS before the experiment. This layer was used to promote the attachment of the second layer. For the second layer, around 10,000 cells mixed with 80 lL of 1% LMA (pH 7.4) were rapidly pipetted onto this slide, spread using a cover slip and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the cover slip, the slides were immersed in cold lysing solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} Na_2EDTA, 10 mmol L^{-1} Tris, 1% sodium sarcosinate, pH 10) with 1% Triton X-100 and 10% dimethylsulfoxide added just before use, for a minimum of 1 h at 4°c

Electrophoresis:

The slides were removed from the lysing solution, drained and placed in horizontal gel electrophoresis tank side by side, avoiding spaces and with the agarose ends facing each other, nearest the anode. The tank was filled with fresh electrophoresis solution (1 mmol

L^{-1} Na_2EDTA 300 mmol L^{-1} NaOH, pH 13) to a level approximately 0.25 cm above the slides.

Tail moment:



Tail moment of Group1: Clearfil SE bond, Group 2:Gluma bond, Group 3:Futura bond treated human peripheral lymphocytes.
Where as G1:Group 1;G2:Group 2; G3:Group 3

Before electrophoresis, the slides were left in the solution for 20 min at 4°c to allow the unwinding of the DNA and expression of alkali labile damage.

Electrophoresis was conducted at a low temperature (4°c) for 20 min using 24 V and adjusting the current to 300 mA by raising or lowering the buffer level and using a compact power supply (Power Pack P 25 Biometra Analytic GmbH). All of these steps were conducted under dimmed light to prevent the occurrence of additional damage. After electrophoresis, the slides were taken out of the tank, washed in distilled water. Tris buffer (0.4 mol L^{-1} Tris, pH 7.5) was added drop wise and gently to neutralize the excess alkali, and the slides were allowed to sit for 5 min. The neutralizing procedure was repeated thrice. After waiting for the slides each for 5 min in distilled water, 50%, 75%, and 99% ethanol, they were allowed to dry at room temperature.

Staining and slide scoring:

30 lL of EtBr (20 lL mL^{-1}) was added for each slide. Then the slides were examined at a 1000x magnification using a 40x objective on a fluorescence microscope Leica (Wetzlar, Germany) to visualize DNA damage.

Comet V image analysis software was developed by kinetic imaging was used to assess the extent of DNA damage in the cells. The length of DNA migration and the percentage of migrated DNA were measured by this.

Breaks in the DNA molecule disturb its complex super coiling, allowing liberated DNA to migrate towards the anode. Staining shows the DNA as 'comets'. The mean value of the tail length, tail intensity and tail moment was calculated and used for the evaluation of DNA damage.

Statistical analysis

- Results were expressed in terms of Mean and SD
- Comparison of mean and SD was done in all three groups together by using One way ANOVA test
- Mean difference significance was seen by using Tukey's post hoc HSD test

Results:

Cell viability, as tested using trypan blue dye exclusion of each treated group, was more than 90%. The DNA damage expressed as tail length, tail intensity and tail moment in the lymphocytes. According to data obtained from 3 separate experiments. Tail Length and tail intensity were significantly increased by 1.78 and 49.5 respectively at a concentration of 2.5mgml concentrations of clearfil SE bond, gluma bond, and futura bond compared to untreated cells (fig1 & fig 2). Tail moment was significantly increased ($p<0.00l$) above the control values at 1.25mgml, 2.5mg ml conc. of Clearfil SE bond, 2.5mg ml of gluma bond, 2.5mg ml of futura

bond as compared with untreated cells.(fig 3). No significant increase in DNA damage in the lymphocytes was observed with all the concentrations of gluma bond and futura bond.

Discussion:

It has been observed that, certain components of composites and bonding materials influence adverse effects because they may be released into the saliva during implantation and even after polymerization and diffuse into the tooth pulp, gingiva or mucosa, and salivary gland and causing cytotoxicity in pulp cells via the generation of reactive oxygen species that may also contribute to genotoxic effects.[8] The existing data on the genotoxic effects of dentin bonding agents on human cells are limited and controversial.

Only a few studies evaluated the genotoxic potential of dental adhesive systems using a comet assay on human blood cells within last decade. Therefore, it is difficult to compare the results of this study to other investigations. Several studies tested the genotoxic effects of isolated monomers, in attempts to identify those responsible for genotoxicity. However, this is not similar to a clinical situation. Concentrations needed to elicit reactions in mutagenicity experiments are higher than those expected in materials that are used on patients. In vivo investigations with quantitative measurements of monomers released from humans are lacking, but a series of studies conducted on guinea pigs determined that despite, using a high administered dose of TEGDMA, peak TEGDMA levels in all tissues examined after 24 hours were at least 105 fold less than known toxic levels.[15]

Schweikl et al. stated that dose-related increase in the numbers of micronuclei was also observed with TEGDMA, HEMA, and GMA, suggesting a clastogenic activity of these chemicals. Due to the high concentrations of Methyl Methacrylate and Bisphenol A very low activity of BIS-GMA and UDMA and the elevated numbers of the micronuclei are caused and are associated with cytotoxicity. It is shown that in the mammalian cells the deletions of DNA sequence and gene mutations are also caused by TEGDMA.[12]

Dental adhesives have a considerably fair amount of biocompatibility as they are in long term and direct contact with the oral tissues. After eluting polymerized dental adhesives (Excite, Adper single bond 2, prompt L-Pop and Optibond solo plus) in dimethylsulfoxide for 1hr, 24hrs, and 120hrs, genotoxicity was evaluated by micronucleus test. At the end of 1hr only, the highest dose of all tested materials affected the measured cytogenetic parameters. After 24hrs, genotoxicity was demonstrated only in cultures treated with elutes in a concentration of 0.5 µg/mL and 5 µg/mL. Studies by Prica D et al. concluded that genotoxicity was caused in human lymphocytes. Toxic effects of dental adhesives are directly proportional to the concentration and inversely to the length elution period. [10]

Correlative to present findings of the study, the methacrylates TEGDMA, UDMA, BIS-GMA, and HEMA shows significant DNA migration in higher concentrations in human lymphocytes as human target cells of carcinogenesis. In higher concentrations, all tested substances induced significant, but minor enhancement of DNA migration in the comet assay as a possible sign of limited genotoxic effects. At concentrations possibly relevant for the in-vivo situation $(<10)^4$ mol $L)^1$), there was no significant enhancement of DNA migration in the comet assay. [6,7]

In the present study, Gluma bond and futura bond caused DNA damage at concentrations of 1.25mg ml and 2.5 mg ml in human lymphocytes, whereas Clearfil SE Bond induced DNA damage only at the higher concentrations of 2.5mg mL) compared to controls. For the positive control, H_2O_2 is used because it induces DNA damage without cytotoxic effect.[6,7] The DNA damage induced by the H_2O_2 is higher than the DNA damage observed with the dentin bonding agents. The differences in the genotoxic effects have been observed from the results of various components in the formulas. But, it can be seen that it is possible to get a genotoxic effect of dentin bonding materials in a dose manner such as Clearfil SE bond; however, it is impossible to find the causative agents.[5]

Conclusion: It can be concluded that Sixth generation dentin bonding agents (Clearfil SE bond) increased Tail length and Tail intensity of DNA damage in human peripheral lymphocytes in higher doses i.e, 2.5mg ml concentration when compared to Seventh (Gluma bond) and Eighth generation (Futura Bond) dentin bonding agents.

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