INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

"DETERMINATION OF GENOTOXICITY OF DENTINE BONDING AGENTS"

Dental Science

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ABSTRACT

Aim: The aim of the present study was to assess the genotoxicity of the newer adhesives and primers, which is essential since these are directly used on vital dentin in most clinical situations. Genotoxicity damage may significantly diminish the self repairing potential of the tissue in long term.
METHODOLOGY: Human gingival fibroblasts were isolated from healthy gingival tissue biopsies of patients. These cells were cultivated in 25cm^3 tissue culture flasks. All the test materials were dispensed aseptically and serial dilutions for each compound tested were prepared. The elutes at all concentrations were obtained at 48 hours for each material and were added to the Human gingival fibroblasts (HGF) cultures. This was then followed by a single cell gel electrophoresis technique (SCGE) to check for genotoxicity of only the three bonding systems. The percentage of cell viability and the length of the comet cells for each group was recorded
CONCLUSION: In conclusion none of the three bonding systems tested in this study completely eliminated to be genotoxic at the tested concentrations.

KEYWORDS

Genotoxicity, Biocompatibility, self etch primers, total etch primers

INTRODUCTION

Adhesive resins bond to the enamel well and exhibit very high bond strengths. R.L.Bowen's discovery of resin's bonding to enamel was strengthened by the discovery of acid etch technique in the year 1955 by Buonocore.

When the dentin resin composite and the resin based dentin bonding systems are used clinically and cured under clinical conditions it results in free resin monomers due to incomplete polymerization. It is impossible to expect 100% conversion of monomers to polymers. The left over free monomers can enter deeper into the dentin pulp system. "In genetics, genotoxicity describes the property of chemical agents that damages the genetic information within a cell causing mutations."[1]

To assay for genotoxic molecules, researchers assay for DNA damage in cells exposed to the toxic substrates. Consequently, many sophisticated techniques like Ames Assay and Comet Assay have been developed to assess the chemicals potential to cause DNA damage that may lead to cancer.

Comet Assay is the commonly preferred technique as it has an advantage of detecting low levels of DNA damage, requires a very small number of cells, inexpensive than many techniques, easy execution, and quick display of results.[2]

Modern day dentistry has seen the dawn of the development of monomeric resinous materials and dental adhesive systems improving the contact between the restorative material and the walls of the prepared cavity of the tooth.

The resinous monomers used in dentistry are formed by different organic molecules, such as bisphenol A-glycidyl methacrylate (Bis-GMA), triethylene glycol dimethacrylate(TEGDMA), urethane dimethacrylate (UDMA) and hydroxyethyl methacrylate(HEMA), which work together as co-polymeric chains and are the main organic basis of the majority of resins and dental adhesives.[3]

The production of reactive oxygen species (ROS) indicates an onset of processes leading to cell death via apoptosis and DNA damage with an elevated numbers of micronuclei. Thus proving them to be genotoxic.[4]

The purpose of the study was to compare the response of cultured human gingival fibroblasts to genotoxic substances leached from a conventional (total etch) and a self-etching adhesive system.

MATERIALS AND METHODS

1. Materials

Three commercially available dentin bonding systems were included; two of them being total etch systems and third one a self etch adhesive. The total etch bonding systems included were Clearfil SE Bond (*Kuraray Noritake Dental Inc. Okayama, Japan*) which is commercially available as two bottle system, one being the primer and the other one a bonding agent and Clearfil Protect Bond (*Kuraray Noritake Dental Inc. Okayama, Japan*) also available as two bottle system of a primer and a bonding agent. The third system in comparison was a self etch adhesive One Coat 7.0 (*Coltene/ Whaledent AG, Altstatten, Switzerland*) which is single bottle system. The composition of the dentin bonding systems used is shown below.(Table 1)

TABLE 1:

Dentin Bonding Agent	Composition	Percentage By Weight/ Density
Clearfil SE Bond	10-Methacryloyloxydecyl dihydrogen phosphate (MDP), Bis-GMA, Hyrdoxyethylmethacrylate (HEMA) Hydrophobic aliphatic methacrylate Colloidal silica, D,L-camphorquinone	25-45% 20-40%
Clearfil SE Primer	10-Methacryloyloxydecyl dihydrogen phosphate (MDP), Hyrdoxyethylmethacrylate (HEMA), Hydrophobic aliphatic methacrylate D,L-camphorquinone, Water	10-30%
Clearfil Protect Bond	10-Methacryloyloxydecyl dihydrogen phosphate (MDP), Bis-GMA, Hyrdoxyethylmethacrylate (HEMA) Hydrophobic aliphatic dimethacrylate D,L-camphorquinone, N,N-diethanol p-toludine Silanated colloidal silica, Sodium Fluoride	25-50% 25-50% ≤ 2.5%
Clearfil Protect Primer	10-Methacryloyloxydecyl dihydrogen phosphate(MDP), 2-hydroxyethyl methacrylate, 12-Methacryloyloxydo, decylpyridinium bromide, Hydrophilic aliphatic dimethacrylate, Water	25-50%
One Coat7.0	Hydroxyethylmethacylate, Ethanol	<30% <40%

2. Method of study

Three commercially available dentin bonding systems were investigated. The primer and bond of each in the total etch systems were taken as separate groups.(TABLE 2)

The single cell gel electrophoresis (SCGE)/ comet assay was used that combines the simplicity of biochemical techniques for detecting DNA single strand breaks.

The advantages of the SCGE technique include:
a) The collection of data at the level of the individual cell, allowing for more robust types of statistical analysis;
b) The need for only small numbers of cells per sample (<10,000);
c) Its sensitivity for detecting DNA damage
d) Virtually any eukaryotic cell population is amenable to analysis.

A working protocol that can be used to detect DNA damage is given below.[5]

Preparation of base slides
1%, 0.5% Low Melting Point Agarose (LMPA) and 1.0% Normal Melting Agarose (NMA) were prepared. For LMPA, 5 mL samples were aliquot into scintillation vials and refrigerated until needed.

The slides were dipped in methanol and burnt over a blue flame to remove the machine oil and dust. Care was taken to avoid high humidity conditions and stored at room temperature until needed. Slides were prepared a day before the use.[5]

Cell cultures:
a. Growth and Maintenance of Cells:
Human gingival fibroblasts were isolated from healthy gingival tissue biopsies (explants). The tissue was washed in phosphate buffer saline and minced into small pieces of approximately 0.3 cm in size.

The explants were cultivated in 25 cm^3 tissue flasks containing Dulbecco's modified Eagle's Medium (DMEM) (*HiMedia Labs, India*). Sub cultivation was performed with cell cultures treated with Trypsin. The cells were then counted using Tryphan Blue Dye on a Nuebaer's Chamber, seeded at a density of 25,000 cells per well in the 96 well plate and finally incubated for 24 hours, to get sub confluent monolayers of cells*.*

b. Sample preparation and elution
All the test materials were dispensed aseptically according to manufacturer's instructions. Serial dilutions for each of these testing components were prepared and placed in cell culture medium Dulbecco's Modified Eagle Medium (DMEM) and sterile filtered before being exposed to the culture. DMEM culture medium alone was used as control.

c. Addition of elutes or extract to cells in culture:
There were 5 experimental groups as follows:

TABLE 2

Group 1	Clearfil SE Bond
Group 2	Clearfil SE Primer
Group 3	Clearfil Protect Bond
Group 4	Clearfil Protect Primer
Group 5	One Coat 7
Positive Group	Cells Alone
Negative Group	Hydrogen Peroxide

After incubation for 48 hours, the medium was aspirated from all wells and test solutions were added to 3 wells per material per concentration and one column of wells was filled with only culture medium and another column of wells was tested with hydrogen peroxide as control groups. The plates were incubated for another 24 hours.

d. Monolayer Cultures
The media was removed and replaced with mincing solution, cells were scraped off to yield approximately 1x10 cells/ml.

e. Suspension Cultures
10,000 cells in per 75 µl LMPA were mixed and processed accordingly.

f. Viability Assay
10 µL of at least 106 cells/ml were placed in a microcentrifuge tube, with trypan blue dye. It was allowed to stand for at least two minutes and then placed on a slide and covered using a coverslip.100 cells were scored and the number of viable cells (shiny) and dead cells (blue) were then recorded.

g. Electrophoresis of Microgel Slides
After at least 2 hours at ~4ºC, slides were removed from the Lysing Solution and placed side by side on the horizontal gel box. The buffer reservoirs were filled with freshly made Electrophoresis Buffer of pH>13until the liquid level completely covered the slides.

The slides were kept in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage.

The power supply was turned on to 24 volts and the current was adjusted to 300 MA after which the slides were electrophoresed for 30 mins. The slides were coated drop by drop with Neutralization Buffer, and allowed to sit for at least 5 minutes. Slides were then stained with 80µL Ethidium Bromide, followed by scoring immediately.

h. Evaluation of DNA Damage
For visualization of DNA damage, observations were made of EtBr-stained DNA using a 40x objective on a fluorescent microscope.

A Komet 5 image analysis software linked to a CCD camera was used to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the tail length. 50 to 100 randomly selected cells are analyzed per sample.

RESULTS
The test showed that there was a very high significance in the comet lengths' value among all the groups. However not high significant difference between:

1. Protect Primer (3 mg/ml) and SE Primer (5 mg/ml)
2. Protect Bond (10 mg/ml) and SE Bond (10 mg/ml)

The longest of comet tail lengths were seen in both the concentrations of Clearfil Protect Bond (0.919µm & 0.920 µm respectively) and the shortest lengths were of the lower concentration (0.125mg/ml) from Clearfil Protect Primer group (0.201µm) and the negative control group. One coat showed same lengths in both concentrations (0.551 µm) which were intermediate findings between the shortest and longest tail lengths. (Figs: 1,2 & 3)
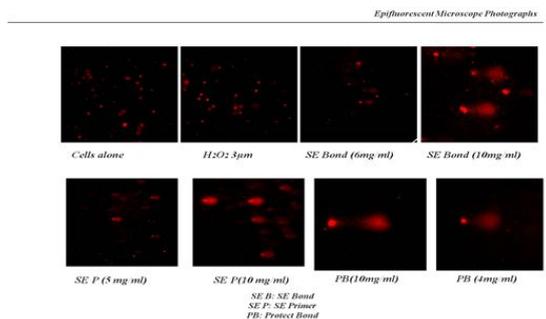

Fig 1: Images of cells with variable levels of DNA damage in comet assay at different concentrations for the control groups, Clearfil SE Bond, Clearfil SE Primer, Clearfil Protect Bond, as taken with a Fluorescent Microscopic.
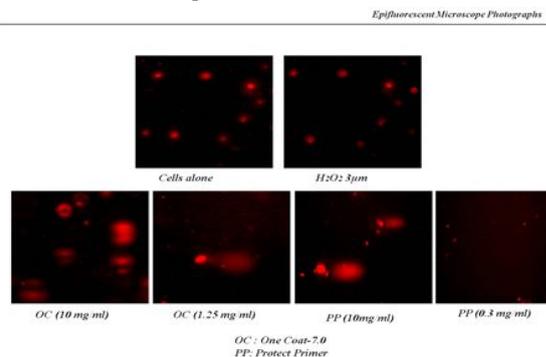

Fig 2: Images of cells with variable levels of DNA damage in comet assay, at different concentrations for the control groups ,One Coat and Clearfil Protect Primer, as taken with a Fluorescent Microscopy.
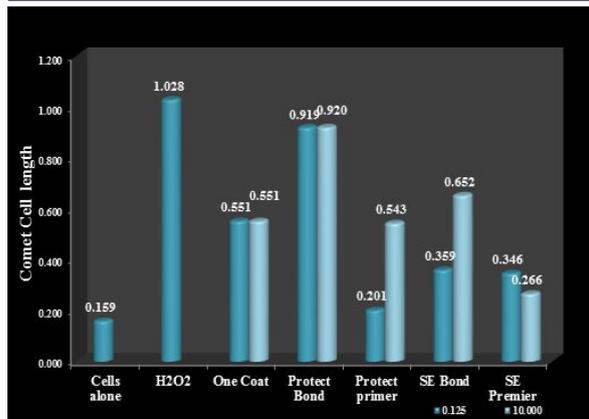
Fig 3: Bar Diagram Showing Comparison Of Mean Values Of Comet Cell Length Between The Experimental Groups And Controls Treated Human Gingival Fibroblasts At Two Concentrations (10 And 0.125 Mg/Ml)

DISCUSSION

The existing data on genotoxic effects are limited and controversial.[6] The chemistry of most of the resin based materials are similar, wherein a large portion of the polimerizable groups do not react and remain unbonded during curing in the final set stage.[7]

Kaya et al (2011) in their study, evaluated the possible genotoxicity of dental bonding adhesives and primers that contain nearly the same resin monomers used in the present study and previously used for testing on human lymphocytes ex-vivo at different elution concentrations by alkaline comet assay.[4]

In their study Kaya et al (2011), suggested that components of composites and bonding materials exerted adverse effects because they may be released into the saliva during implantation and even after polymerization and diffuse into the tooth pulp, gingival, mucosa or salivary glands. As mentioned before, the residual monomers may also contribute to genotoxic effects.[4,8]

In the present study the DNA damage was expressed as comet lengths of the human gingival fibroblasts cells following ex-vivo treatment with two total etching and a self etch bonding system. According to the data obtained; tail length significantly increased ($p<0.001$) at concentrations of 10mg/ml and 0.125mg/ml for Clearfil Protect Bond comparing them with both control groups, indicating high genotoxicity. Followed by Clearfil SE Bond at 10mg/ml, One Coat-7.0 at 10mg/ml and 0.125mg/ml, Clearfil Protect Primer at 10mg/ml, Clearfil SE Bond at 0.125mg/ml, Clearfil SE Primer at 0.125mg/ml and 10mg/ml, the shortest comet lengths indicating lowest genotoxicity with Clearfil Protect Primer at a concentration of 0.125mg/ml.

A dose related increase in the number of micronuclei was observed suggesting a clastogenic activity of these chemicals.[4] Dimethacrylates are used as monomers in dental resinous materials such as TEGDMA and UDMA which were found to be genotoxic in human lymphocytes, however no specific component was identified as genotoxic in these studies.

Kaya et al (2011) [7] in their study using a similar method of genotoxicity evaluation concluded that Clearfil SE Bond primer caused DNA damage in human lymphocytes whereas Clearfil Protect Bond primer induced DNA damage only at higher concentrations compared to controls. A significant increase in DNA damage was also observed with Clearfil Protect Bond and Clearfil SE Bond adhesive in human lymphocytes at higher concentrations. This is in agreement with the data obtained in the present study and these results are also consistent with other studies that have examined genotoxic effects ex-vivo and in-vivo by comet assay.

Moharamzadeh K et al (2009)[9] stated that the biological end point in genotoxicity tests is seen with changes in the DNA or in the chromosomes themselves.

Within the limitations of this in-vitro study; all of the bonding systems

tested demonstrated a varied amount of genotoxic response from lowest to highest toxicity.[10] However a very extensive research is required to come to a conclusion before ranking the order of the cytotoxicity or genotoxicity of materials tested.

CONCLUSION

Under the conditions of this in-vitro study, it can be concluded as None of the three bonding systems tested in this study completely eliminated genotoxicity at the tested concentrations.

The lowest genotoxic results were obtained from Clearfil Protect Bond adhesive group which showed longer comet lengths and higher genotoxic results were obtained from Clearfil Protect Primer group with shorter comet lengths.

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