



Cytotoxic and Genotoxic Effects of Orthodontic Adhesives on Human lymphocyte: An In-vitro Study

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Abstract

Aim of this study was to evaluate the in vitro genotoxicity and cytotoxicity of two orthodontic adhesives and to determine the type of cell death they induce on human lymphocytes. The materials tested were 1. Light cure orthodontic adhesive with conventional primer (Transbond XT3M) and 2. Self cure orthodontic adhesive (Unite, 3M). Cured sterile individual masses were immersed in DMEM and left at 37°C for 24 h. Then a volume of 200 µL of the extract medium was mixed with human peripheral blood lymphocyte tested for comet assay by Single cell DNA Damage assay and Apoptosis by DNA diffusion agar assay. Evaluation of Cytotoxicity was carried out by Hemolysis assay method. Hemolytic activity of Self cure orthodontic adhesive (52.9 ± 1.82) was significantly more than that of Light Cure orthodontic adhesive (48.9 ± 1.23). The results showed all parameters studied by comet assay were significant ($P > 0.05$). In Case of Apoptosis, Light cure orthodontic adhesive (155.11 ± 6.03) and self cure orthodontic adhesives (154.77 ± 13.17) showed slightly increased diffusion of DNA compared to normal lymphocyte (111.22 ± 8.78). However the level of DNA diffusion was not significantly different between the two adhesives. Light Cure orthodontic and self cure orthodontic adhesives were cytotoxic and induced apoptosis. The self cure orthodontic adhesive was found to be significantly more toxic than that of Light Cure orthodontic adhesive. Both the adhesives had no significant effect on the percentage of DNA tail and Tail length of the human lymphocyte.

Key Words: Orthodontic adhesives, DNA damage, apoptosis, lymphocyte

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Introduction

In the selection of dental materials, biological compatibility is of primary importance, as contact or interaction with oral tissues and body fluids may cause local and or systemic adverse effects. It has been reported that dental adhesives release substances that have biological effects and toxic potencies [1, 2].

Generally, in vitro tests using cell cultures provide rapid, sensitive, inexpensive, convenient and repeatable means of screening and ranking materials [3-7]. In most cases, adhesives may come in direct contact with the soft or hard tissues for a prolonged period of time and might affect the surrounding tissues or could also delay healing. Several in vitro tests have been used for the evaluation of the biological effects of adhesives [8].

The use of genotoxicity testing is essential for evaluation of potential human toxicity so that hazards can be prevented [9]. To date, a variety of assays can measure genotoxicity, such as the bacterial reverse gene mutation assay (Salmonella reversion assay or Ames test), the chromatid sister exchange, the mouse lymphoma gene mutation assay, the micronucleus test, the chromosome aberration test and the comet assay [9].

With the evolution of Orthodontics, there has been a transition from banding to bonding technique which is far more superior and thus bonding adhesives came into force. Previous studies have shown that Orthodontic bonding adhesives are cytotoxic and need further investigation [10,11].

Excessive bonding adhesive left around the bracket is under the influence of atmospheric oxygen that compromises its polymerization reaction giving rise to an oxygen inhibited layer of low molecular weight [12]. Also, atmospheric oxygen has a high affinity for free radicals and tends to prematurely terminate the chain reaction of the monomers, leaving behind a layer of short chain hydrocarbon on the adhesive surface [13]. Both these leaching components inside the resin bulk and the oxygen inhibiting layer (OIL) may produce cytotoxic effects which compromise the bond strength [14,15].

In this study, hemolysis assay, apoptosis and the comet test were used for cytotoxicity and genotoxicity testing. The aim of this in vitro study was to evaluate the cytotoxicity and genotoxicity of two orthodontic adhesives applied to human lymphocyte.

Materials and Methods

Orthodontic adhesives

Two Orthodontic adhesives were tested: 1) Light Cure orthodontic adhesive with conventional primer (Transbond XT3M) and 2) Self cure orthodontic adhesive (Unite, 3M). The Adhesives are resin based. Compositions of the adhesives are given in Table 1.

Sample Preparation:

Orthodontic Adhesives of same size were cured according to the manufacturer's instruction and the individual masses were immersed in DMEM (Himedia) and left at 37°C for 24 hr. Then a volume of 200 µL of the extract medium was utilized for study.

Blood Sampling:

Lymphocyte Separation:

Whole Blood was drawn by antecubital venipuncture into heparinized vacutainers. 1:1 ratio of Histopaque (Purchased from Sigma Aldrich) was added and centrifuged at 3000rpm for 10 minutes. Lymphocyte was separated and used for genotoxicity study.

Erythrocyte Separation:

The erythrocytes were collected from the peripheral blood and then washed three times with 0.85% NaCl saline solution. After each washing cells were centrifuged 150g for 5 minutes, supernatant was discarded. Finally 2% erythrocyte suspension was prepared using 0.85% sodium chloride saline.

Cytotoxicity and Genotoxicity

Hemolysis Assay:

This assay was performed as per the method described by Black *et.al.* 2003, with slight modification. The erythrocytes were collected from the peripheral blood and then washed three times with 0.85% NaCl saline solution. After each washing cells were centrifuged 150g for 5 minutes, supernatant was discarded. Finally 2% erythrocyte suspension was prepared using 0.85% sodium chloride saline [16].

Both the adhesives were prepared in phosphate buffer. 200µl of these extract were taken in separate test tubes and volume was made up to 200µl using buffer saline. Tubes were containing distilled water served as control. To this 200µl of erythrocyte were added. After 30 minutes of incubation at 37°C liberated hemoglobin was estimated at 405 nm and percentage of hemolysis was determined (n=2). The percentage of hemolysis was calculated using the formula,

$$H\% = \frac{A_t}{A_a} \times 100$$

A_t = Absorbance before hemolysis; A_a = Absorbance after hemolysis

Alkaline comet assay:

The alkaline comet assay was performed basically as described by Tice *et al.* 1991. Electrophoresis, which allowed for fragmented DNA migration was carried out for 20 min at 25 V and 300 mA. After electrophoresis, the slides were neutralized with 0.4 M Tris, pH 7.4, stained with 50µL of ethidium bromide (20µg/mL) and analyzed with a fluorescence microscope (Olympus.40x objective). The extent of DNA damage was assessed from the DNA migration distance, which was derived by subtracting the diameter of the nucleus from the total length of the comet. Fifty randomly selected cells were examined for each replicate, for each sample or subject. The quantification of the DNA strand breaks of the stored images was performed using Comet score software by which the percentage of DNA in the tail, tail length and OTM could be obtained directly [17].

Apoptosis assay:

Apoptosis assay was performed basically as described by Singh *et.al.* 2004. The “DNA diffusion” assay described here is a simple, sensitive, and rapid method for estimating apoptosis in single cells. The assay involves mixing cells with agarose and making a microgel on a microscopic slide, then lysing the embedded cells with salt and detergents (to allow the diffusion of small molecular weight DNA in agarose), and finally visualizing the DNA by a sensitive fluorescent dye, ethidium bromide [18].

Table 1. Compositions of orthodontic adhesive systems.

Material	Composition
Transbond XT-Light Cure Kit (3 M Transbond XT, India)	Benzoin ethylether, camphorquinone, Dimethylaminoethyl-methacrylate (DEAEMA 0.15%), organosilanes, zirconates, titanates
Unite Bonding Kit-Self cure (3 M Unite, India)	Monomers- BIS-GMA, UDMA, Viscosity controllers- DEGMA, TEGDMA, Inhibitors- 4 methoxy phenol (PMP), 2,4,6 tritertiary butyl phenol, phenylsalicylate, Benzoyl peroxide, Tertiary amine(dihydroxy ethyl p toludine)

Results

Hemolysis Assay

Hemolysis assay is an extremely sensitive method for cytotoxic studies. We observed significant haemolytic activity of dental adhesives. Self cure orthodontic adhesives (52.9 ± 1.82) haemolytic activity was slightly more than that of Light Cure orthodontic adhesive (48.9 ± 1.23). The percentage of haemolysis showed in Figure 1.

Apoptosis Assay

The “DNA diffusion” assay described as a simple, sensitive, and rapid method for estimating apoptosis in single cells. Figure 2 shows the result of Diffused DNA diameter determination in lymphocyte mixed with Dental adhesives. Light cure orthodontic adhesive (155.11 ± 6.03) and self cure orthodontic adhesives (154.77 ± 13.17) showed slightly increased diffusion of DNA compared to normal lymphocyte (111.22 ± 8.78). Apoptotic DNA is showed in Figure 3.

Alkaline comet assay

To investigate the effect of EBR induced DNA damage in lymphocyte, single cell gel electrophoresis was performed. Table 2 shows the results of comet parameter determination on dental adhesives mixed with lymphocyte. There is significant increase in the Tail length and percentage of tail DNA in Transbond light cure compared with normal lymphocyte and self cure orthodontic adhesive.

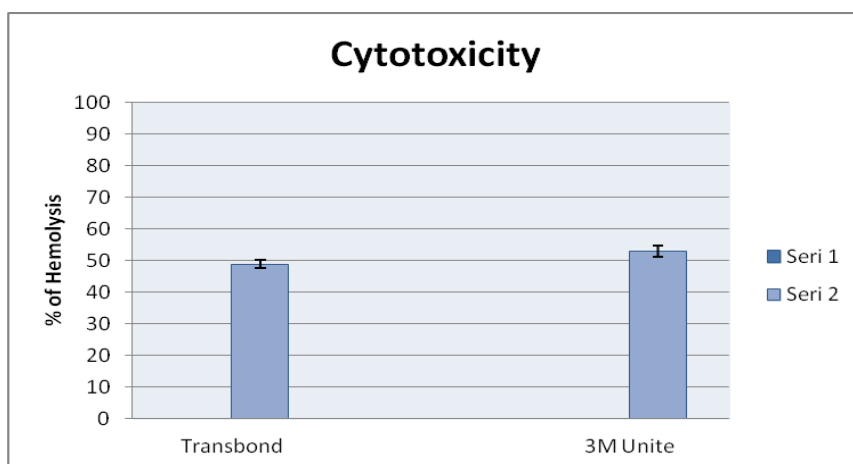


Figure 1. The percentage of hemolysis of two orthodontic adhesives.

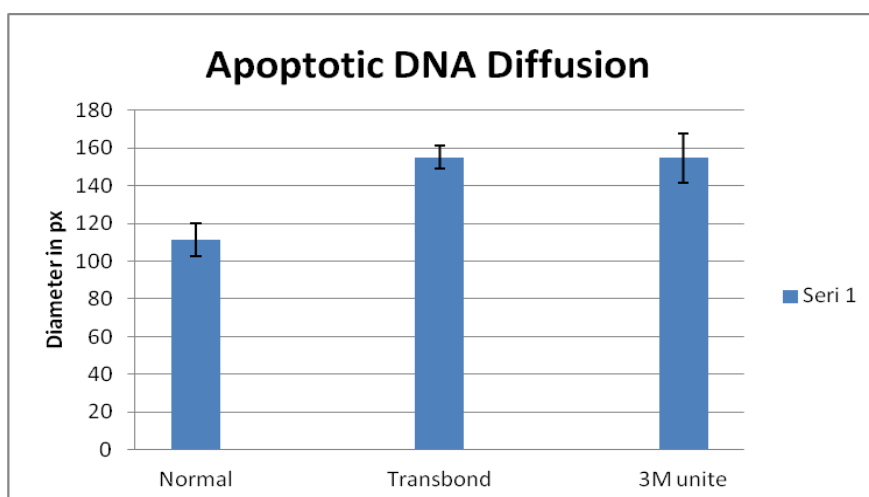


Figure 2. Apoptotic DNA Diffusion of Light cure and Self Cure Orthodontic adhesives.

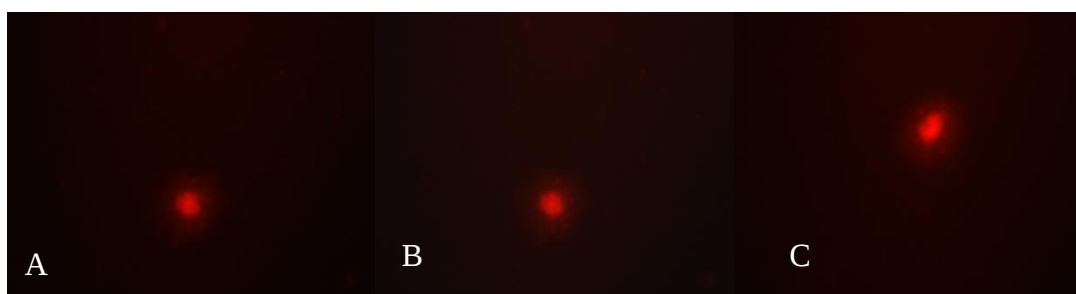


Figure 3. Depicts of Images of Apoptotic Cells. Image A: Normal Cell, Image B: Apoptotic Lymphocyte DNA mixed with Transbond light cure orthodontic adhesive. Image C: Apoptotic Lymphocyte DNA mixed with Self Cure orthodontic adhesive.

Transbond light cure (3.86 ± 0.85) shows slightly increased olive moment compared to normal lymphocyte (1.83 ± 0.23) and self cure orthodontic adhesive (3.54 ± 0.62) is shown in Figure 4.

Table 2. Percentage of DNA in tail and tail length of lymphocyte DNA damage.

	Tail Length (px)	%DNA in Tail
Normal	82.90 ± 8.32	6.90 ± 1.28
Transbond	122.83 ± 13.28	11.62 ± 1.81
3M Unite	105.5 ± 11.23	8.23 ± 1.14

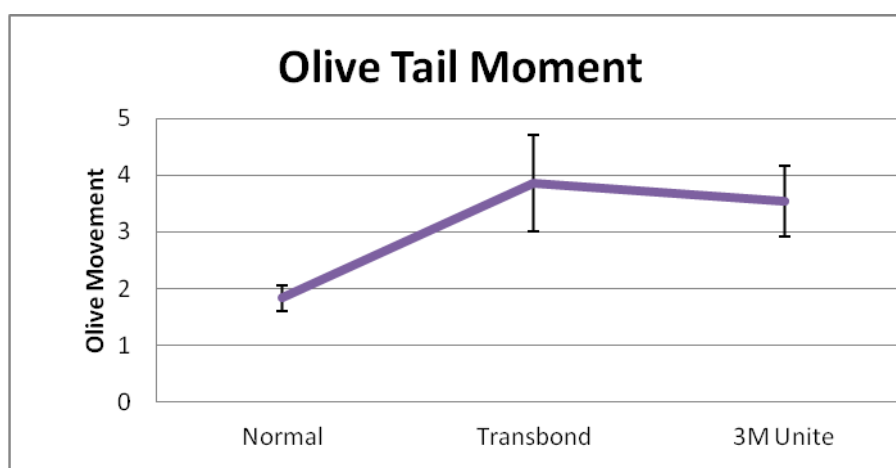


Figure 4. Olive tail moment of light cure and self cure orthodontic adhesives.

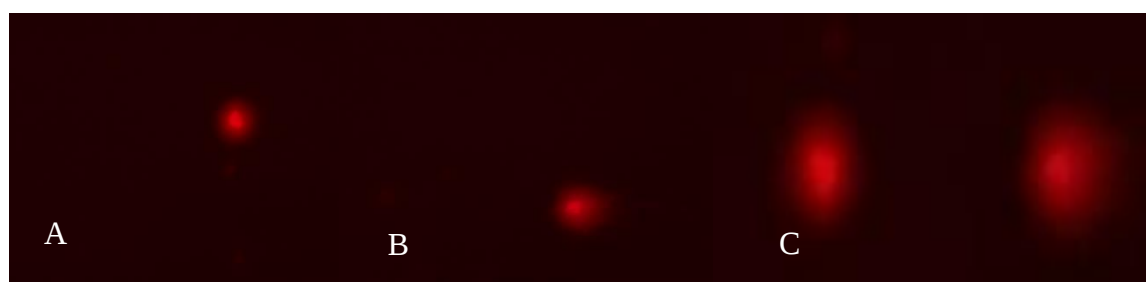


Figure 5. Depicts of Images of DNA Damage by Comet assay. Image A: Normal DNA, Image B: DNA Damage showed by lymphocyte mixed with adhesive. Image C and D: DNA Damage showed by lymphocyte mixed with Self Cure orthodontic adhesive.

Discussion

Biocompatibility testing of materials that come in close contact with normal tissues is crucial for the quality of host-to-graft acceptance. Assays measuring cytotoxicity and genotoxicity are a critical part of testing materials designed for application on human tissues [1].

In the present study, the cytotoxic and genotoxic effects of Light cure and Self Cure Orthodontic adhesives were evaluated on human lymphocyte and erythrocyte in vitro. The differential cytotoxicity of the materials tested could be attributed to the different ingredients, the interactions between them and the degree of resin polymerization. It is known that oxygen acts as an inhibitor of monomers' polymerization. It has also been reported that unfilled resin cured in room air has a significantly greater thickness of polymerization inhibited material than resin cured in an argon atmosphere [19]. The inhibition layer thickness varies across dentin adhesives and depends on the type and combination of monomers existing in each product. In addition, an aqueous environment may interfere with the polymerization of resinous materials [20].

For comet assay, cells should be exposed to the test substance for 3–6 h (Tice et al. 2000). In the present study, the materials tested were placed in direct contact with lymphocytes for 3 h. Direct contact between the adhesives and the lymphocytes simulates the clinical condition. Cytotoxicity and genotoxicity evaluation was performed, enabling the assessment of early and late toxic effects of the materials and the recovery of cells.

Apoptosis is a programmed physiological process of cell death which plays a critical role not only in normal development, but also in the pathology of a variety of diseases and the activity of a large number of toxicants. The mechanisms leading to apoptosis have been extensively reviewed previously. In contrast to apoptosis, necrosis generally sets off a tissue inflammation process associated with clinical symptoms [21].

Conclusion

Light Cure orthodontic and self cure orthodontic adhesives were cytotoxic and induced apoptosis. The self cure orthodontic adhesive was found to be significantly more toxic than that of Light Cure orthodontic adhesive. Both the adhesives had no significant effect on the percentage of DNA tail and Tail length of the human lymphocyte.

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