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Cytotoxic and Genotoxic Evaluation of Three Orthodontic Bonding Adhesives Exposed to Electron Beam Radiation.

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ABSTRACT

To evaluate the in vitro genotoxicity and cytotoxicity of three Orthodontic Bonding adhesives. The materials tested were Self cure orthodontic adhesive (Unite, 3M), and Light Cure orthodontic adhesive with Moisture insensitive Primer(MIP 3M) and Glass ionomer light Cure orthodontic adhesive (GC Fuji).Cured sterile individual masses were exposed to 2kGy electron beam radiation, immersed in Phosphate buffer saline and left at 37^0C for 24 hr. 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. Both irradiated and non-irradiated adhesives showed haemolysis. There is no significant difference in case of Unite 3M, whereas MIP and GC Fuji showed more haemolytic activity in case of after irradiation using Electron beam radiation. In case of DNA Damage parameters there is significant decrease in the Tail length of MIP and GC Fuji after irradiation compare to before irradiation, whereas the percentage of tail DNA was increased in case of after irradiation compare to normal and before irradiated Orthodontic bonding adhesives. In Case of Apoptosis, irradiated group showed increased DNA diffusion compare to non-irradiated group. This study is an attempt to reduce the cytotoxic and genotoxicity of Orthodontic bonding adhesives, used regularly in the treatment of patients. Within the limitations of the present study, it is concluded that further research is needed in the field of electron beam irradiation of dental orthodontic bonding adhesives.

Keywords: Orthodontic Adhesives, Electron Beam Radiation, DNA Damage, Apoptosis.

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INTRODUCTION

Biocompatibility of Orthodontic materials is an important consideration for the patient, clinician, laboratory technician, and manufacturer. It should contain no toxic, leachable, or diffusible substances that can be absorbed into the circulatory system, causing systemic responses, including teratogenic or carcinogenic effects [1].

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 or systemic adverse effects. It has been reported that dental adhesives release substances that have biological effects and toxic potencies [2].

In the field of biomaterials, it is necessary to consider aspects of biosecurity, such as elimination of cytotoxicity and other harmful effects of the material to be used.[1] By definition, the cytotoxicity of an agent means the toxicological risks caused by a material or its extract in a cell culture [2].

Generally, in vitro tests using cell cultures provide rapid, sensitive, inexpensive, convenient and repeatable means of screening and ranking materials [3-5]. 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 [6].

The use of genotoxicity testing is essential for evaluation of potential human toxicity so that hazards can be prevented [7]. 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 [7].

Electron beam processing or electron beam irradiation (EBR) is a process which involves using electrons, usually of high energy, to treat an object for a variety of purposes. This may take place under elevated temperatures and nitrogen atmosphere. Possible uses for electron irradiation include sterilization and to cross- link polymers.

In polymers, an electron beam may be used on the material to induce effects such as chain scission (which makes the polymer chain shorter) and cross linking. The result is a change in the properties of the polymer which is intended to extend the range of applications for the material [8].

The main aim of this study was to evaluate the in vitro cytotoxicity and genotoxicity of orthodontic adhesives.

METHODS AND MATERIALS

Orthodontic adhesives

Three Orthodontic adhesives were tested:

- Self-cure orthodontic adhesive (Unite, 3M).
- Light Cure orthodontic adhesive with Moisture insensitive Primer(MIP 3M)
- Glass ionomer light Cure orthodontic adhesive (GC Fuji)

Compositions of the adhesives are given in Table No.1.

Table No 1: Compositions and components of dental adhesive systems used in the current study.

Material	Composition	Manufacturers
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, phenysalicylate , Benzoyl peroxide, Tertiary amine(dihydroxy ethyl p toludine)	3 M, India
Light Cure orthodontic adhesive with Moisture insensitive Primer(MIP)3M	Methaacrylate –Functionalized polyalkenoic acid co-polymer.	3 M, India
Glass ionomer light Cure orthodontic adhesive (GC Fuji)	Polycarboxylic acid copolymer, 2-hydroxy ethyl methaacrylate, Saline treated glass, Potassium persulfate.	GC Fuji, India

Sample Preparation

Orthodontic Adhesives of same size (8x2x2mm) were cured according to the manufacturer's instruction.

Irradiation of Bonding adhesives

The irradiation work was carried out at Microtron centre, Mangalore University, Mangalore, Karnataka, India.

The Bonding adhesives were placed in polypropylene (PP) tubes and exposed to electron beam at a distance of 30 cm from the beam exit point of the Microtron accelerator at a dose rate of 72 Gy/min. Samples were irradiated at the pulse rate of 100Hz.

Phlebotomy

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 1500rpm

for 5 minutes, supernatant was discarded. Finally 2% erythrocyte suspension was prepared using 0.85% sodium chloride saline.

Direct exposure of Leachable components on Human Lymphocytes

Two different Orthodontic adhesives were prepared i.e, 1.Non-Irradiated and 2. Irradiated (2KGy), Orthodontic adhesives were immersed in Phosphate buffer saline (PBS) overnight at 37^0 C for the release of extract. Lymphocyte was isolated from Whole Blood using Histopaque. 200µl of released extract mixed with equal volume of Lymphocyte and kept for 3hrs in room temperature. Genotoxicity Studies were carried out using the sample or extract.

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 1500rpm for 5 minutes, supernatant was discarded. Finally 2% erythrocyte suspension was prepared using 0.85% sodium chloride saline [9].

Both the adhesives (Irradiated and Non-irradiated) 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 37^0C liberated haemoglobin was estimated at 540 nm and percentage of hemolysis was determined (n=2). The percentage of hemolysis was calculated using the formula,

$$H\% = At / A\alpha \times 100$$

At =Absorbance before hemolysis; Aα =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 24 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 U-LH50HG, 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 [10].

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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 [11].

Statistical Analysis

All values were expressed as Mean ± SD. Comparison between the control and Orthodontic materials were performed by analysis of variance (ANOVA) with Tukey's Multiple Comparison test using Graph pad Prism v3.0 software.

RESULTS

Hemolysis Assay

Hemolysis assay is an extremely sensitive method for cytotoxic studies. We observed significant haemolytic activity of orthodontic adhesives in both the irradiated and non-irradiated samples. There is no significant difference in case of before and after irradiation using Electron beam radiation. The percentage of haemolysis showed in Figure 1.

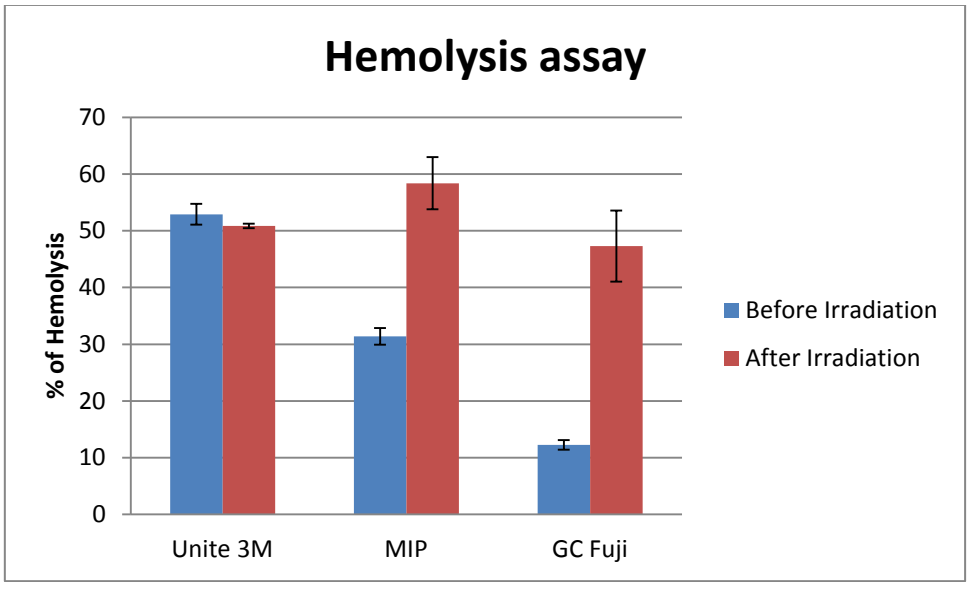

Figure No 1: The percentage of hemolysis of three Orthodontic adhesives (n=2).

Alkaline comet assay

To investigate the effect of EBR induced DNA damage on Orthodontic bonding adhesives, single cell gel electrophoresis was performed. Table 2 shows the results of comet parameter determination of orthodontic adhesives mixed with lymphocyte before and after

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exposed to EBR. There is significant decrease in the Tail length of MIP and GC Fuji after irradiation compare to before irradiation. Whereas the percentage of tail DNA was increased in case of after irradiation compare to normal and before irradiated Orthodontic bonding adhesives.

Table No 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
Unite 3M	105.5±11.23	8.23±1.14
MIP	108.32±6.73	7.86±1.80
GC Fuji	112.67±12.96	8.56±1.43
Unite 3M After Irradiation	109.42±70.32	21.66±13.76
MIP After Irradiation	71.50±26.58	31.17±14.5
GC Fuji After Irradiation	83.36±53.06	15.85±9.11

Figure No 2 show the olive tail moment (OTM). OTM gives an indication of the initial DNA damage and repair after radiation. Figure No 3 depicts the images of DNA Damage by alkaline comet assay.

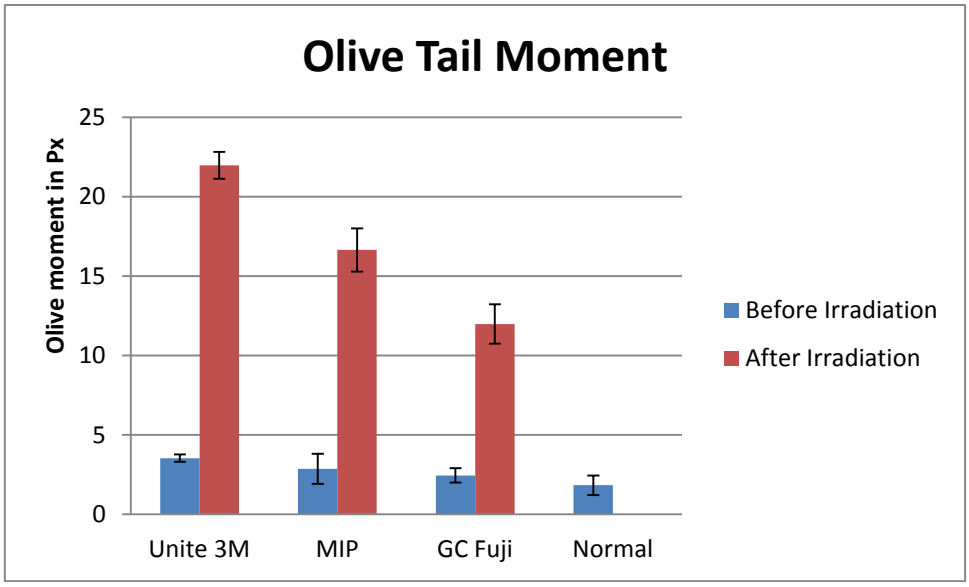

Figure No 2: Olive tail moment of Orthodontic adhesives.

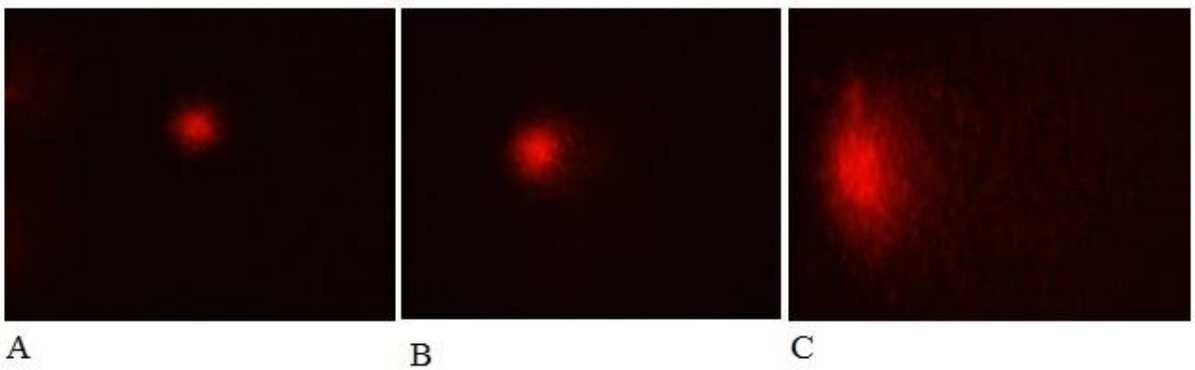

Figure No 3: Depicts of Images of DNA Damage by Comet assay. Image A: Normal DNA, Image B: Slightly Damaged DNA. Image C: Fully Damaged DNA.

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Apoptosis Assay

The "DNA diffusion" assay described as a simple, sensitive, and rapid method for estimating apoptosis in single cells. Figure 4 shows the result of Diffused DNA diameter determination in lymphocyte mixed with orthodontic adhesives before and after exposed electron beam radiation. Apoptotic DNA is showed in Figure 5.

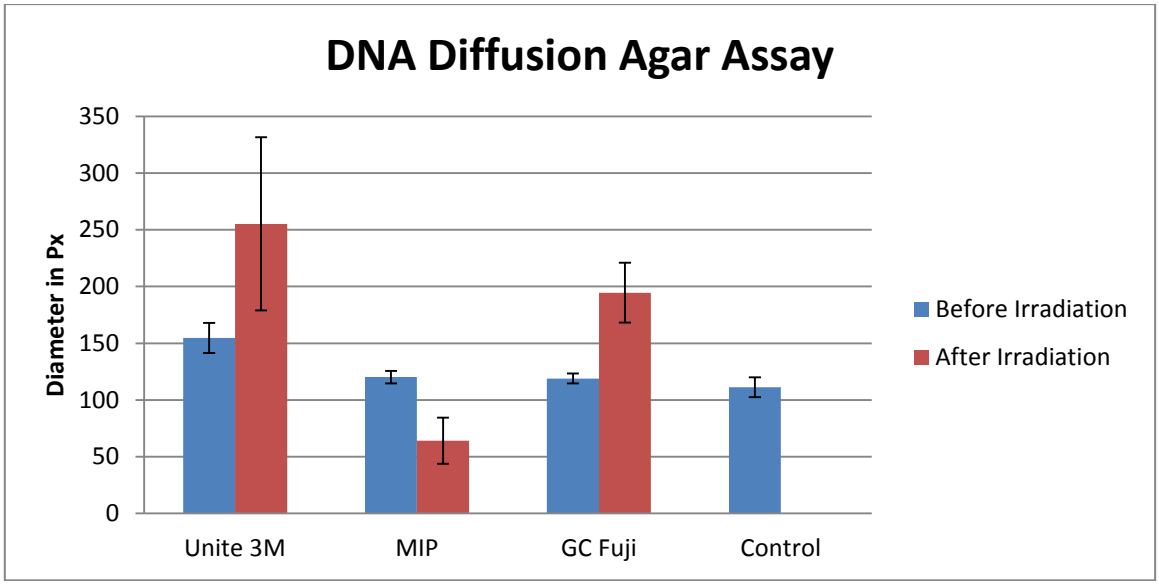

Figure No 4: Apoptotic DNA Diffusion of Orthodontic adhesives.

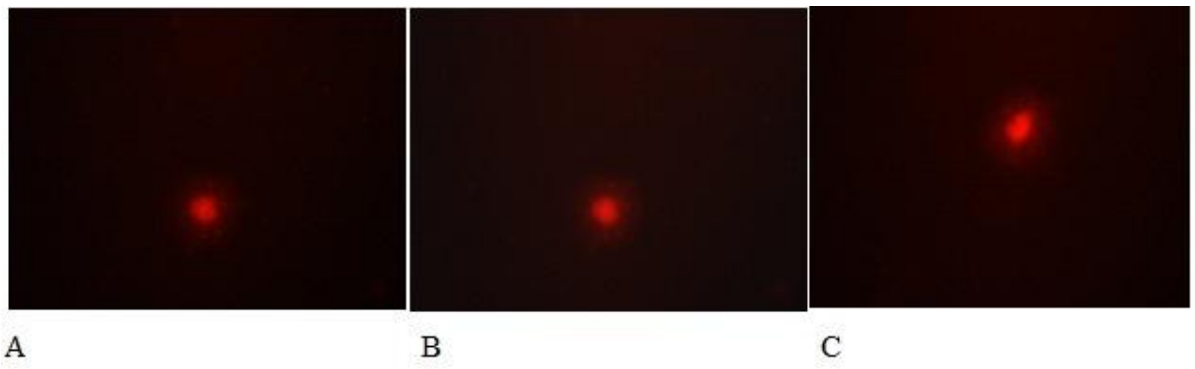

Figure No 5: Depicts of Images of Apoptotic Cells. Image A: Normal Cell, Image B and C: Apoptotic cells.

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 [12].

Bonding in orthodontics, to a great extent has reduced the bulk of fixed orthodontic attachments, thus enabling the patient to maintain the oral hygiene better and thereby drastically reducing the plaque accumulation and gingival inflammation.

By definition, the cytotoxicity of an agent means the toxicological risks caused by a material or its extract in a cell culture [13]. There in the field of biomaterials, it is necessary to consider aspects of biosecurity, such as elimination of cytotoxicity and other harmful effects of the material to be used [11].

The present study is an attempt to evaluate the cytotoxicity and genotoxicity of three Orthodontic bonding adhesives exposed to electron beam radiation.

The concentration of a toxic material to produce hemolytic activity is generally an order of magnitude higher than that required to produce a response in tissue culture, hemolytic activity is considered an important indicator of leachable toxic components.

Hemolytic activity has been highly correlated with both tissue culture response and *in vivo* acute toxicity. Tissue culture and hemolysis in *vitro* tests are reported to provide the most information regarding acute toxicity and are also the most sensitive to variables in the test material formulation**.** Unlike the tissue culture system, the hemolysis assay system is relatively unaffected by secondary time-dependent processes since there is: 1) a low oxidative metabolism in erythrocytes; 2) an absence of metabolic activity associated with growth and reproduction; and 3) the assay time for the tissue culture system is much longer (e.g. for test material-ce1l contact and tetrazolium staining for MTT activity) than that required for the hemolysis assay.

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.

Polymerization processes of dental resin-based materials are usually incomplete under clinical conditions, and almost every component can be detected in extracts of polymerized materials, even when mixed and cured according to the manufacturer's instructions. Leaching from resin composites may occur at two different times: during the setting period of the resin and later when the resin is degraded [14].

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. [15] 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 [16].

A great variety of different test methods are used to determine the risk of such damage to ensure material compatibility. However, the results of such evaluations are dependent not only on the tested material, but also on the test method used. Evaluation of the biocompatibility of dental materials is complex and comprehensive because unwanted tissue reactions may occur in a great variety of types [17].

The present showed no significant difference in case of Unite 3M, whereas GC Fuji and MIP showed significant increase in case of before and after irradiation using Electron beam radiation.

For comet assay, cells should be exposed to the test substance for 3–6 h [10]. 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.

In the present study we observed that the non-irradiated Orthodontic materials showed increased tail length compared to materials with 2KGy Electron Beam radiation. Whereas non-irradiated materials should less percentage of tail length compared to irradited materials.

Olive tail Moment gives an indication of the initial DNA damage and repair after radiation treatment. The present study shows that there is increase in OTM after irradiation compare to the non-irradiated group. The increase in the olive tail moment can be due to direct induction of DNA strand breaks which can be transformed into strand breaks.

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 [18].

Tang et al found out that the viability expressed by both the intact and washed Unite resins, however, demonstrated an increase with time. This suggests that leaching of residual monomers and the associated toxic effect were almost complete by day 1 (after 24 hours)[18]. In the present study we observed after irradiation with electron beam radiation showed slightly increased DNA diffusion compare to before irradiation.

Therefore, the main task for future research is to study the physical and chemical changes occurs in the Orthodontic materials after irradiation with electron beam radiation.

CONCLUSION

This study is an attempt to reduce the cytotoxic and genotoxicity of Orthodontic bonding adhesives regularly used in the treatment of patients.Investigating cytotoxic and genotoxic effects arising from dental materials, including dental adhesive systems is a delicate task. Within the limitations of the present study, it is concluded that further research is needed in the field of electron beam irradiation of dental orthodontic bonding adhesives.

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