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Original scientific paper

EFFECTS OF PHOTOPOLYMERISATION ON GENOTOXICITY OF COMPOSITE ADHESIVES IN THE COMET ASSAY

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Certain *in vivo* studies have shown that the application of adhesives directly onto the open pulp or on a thin layer of dentin causes inflammation and pulpal abscesses. This reaction is related to toxic effects of monomers from adhesives. It has been confirmed that after proper illumination the adhesives become less toxic. The aim of the study was to examine genotoxicity of non-polymerised, partly polymerised and polymerised adhesives on isolated human lymphocytes using the alkaline Comet assay. *Adper Single bond2* and *Adper Easy One/3M ESPE* adhesive photopolymerisation was performed by *Elipar Highlight* 3M ESPE halogen lamp for 0, 10 and 40 sec, at final concentrations of 100, 200, 500 and 1000 μg/mL. With both adhesives, photopolymerisation at 0 and 10 seconds showed statistically significant increase in DNA damage in comparision to the negative control (solvent). On the other hand, after 40 seconds of photopolymerisation of both adhesives in all tested concentrations, the degree of DNA damage in Comet assay had no significant difference ($P>0.05$, χ^2 test) compared to the negative control. Therefore, only the 40 seconds of photopolymerisation prevented genotoxic effects of both adhesives in the Comet assay.

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INTRODUCTION

The use of light-cured composite materials in dentistry has increased during last couple of decades. This increase can be attributed to improvements in formulation, more simplified bonding procedures and increased aestethic demands form patients. Adequate polimerisation is very important for physical properties and safety of resin composites (STANSBURY, 2000). It is well established that dental composite materials are able to come into direct contact with oral tissue, especially gingval cells (TADIN *et al*., 2014). The application of composite adhesives directly on an open pulp or on a thin layer of dentin can cause dilatation and congestion of the blood vessels, inflammation and pulpal abscesses. Non-polymerised monomers from the adhesive are diffused into the pulp through an opening of the chamber or through dentin tubules and cause cytotoxic effects on the cells of the pulp. Complete polymerisation of adhesive resin is impossible during direct closing of the pulp. It has been proven that inappropriate illumination, oxygen, as well as humidity prevent complete polymerisation of monomers from the composites and adhesives. (SOUZA COSTA and MESAS, 2000; CHEN *et al.*, 2001; PASHLEY *et al.*, 2000; MANTELLINI *et al.*, 2003). Toxic monomers are released from inadequately cured resin composites causing cytotoxic and genotoxic effects (KNEŽEVIĆ *et al*., 2008).

There are many studies on the cytotoxicity of various components of adhesives in cell culture (KNEŽEVIĆ *et al*., 2008). The majority of these *in vitro* studies examined which concentrations of some adhesive components caused cytotoxic effects. In addition, possible interactions of some monomers from adhesives (HEMA, Bis GMA, TEGDMA and UDMA) were tested, as well as the length of exposure to these components of mice fibroblasts, lymphocytes and pulp cells (SIDERUDIU and ACHILIAS, 2005). As for the investigations of genotoxic effects, TADIN *et al*. (2014) have found that in addition to cellular damage, the composite materials may cause primary DNA damage in the Comet assay and elevated micronucleus frequency in gingival exfoliated epithelial cells. However, these genotoxic endpoints returned to normal after 180 days of exposure to composite materials, so the observed effects are actually biologically irrelevant.

Toxicity of dental materials can be tested by *in vitro* tests, *in vivo* tests on animals and by clinical tests on humans. *In vitro* tests are primarily conducted for the estimation of cytotoxicity (cell damage) or genotoxicity (for example, DNA damage, gene or chromosomal mutation) of dental material. Contrary to other cytogenetic techniques, Comet assay is not performed on cells in mitosis. It detects primary DNA damage on the level of separate cells. Comet assay is used for determining the presence of single and double strand breaks in DNA, as well as the alkali sensitive sites (apurinic and apyrimidinic sites), DNA-DNA and DNA-protein cross-linking, as well as for studying the phenomenon of DNA repair. Due to its simplicity, high sensitivity and accuracy, Comet assay is used for the assessment of genotoxic potential of various chemical and physical agents (DJELIĆ *et al.*, 2006; DJELIĆ *et al.*, 2007; DJELIĆ *et al*., 2015) and it is also useful for examining dental materials (COLLINS *et al.*, 2004;). The development of single cell gel electrophoresis, (SCGE), also known as the Comet assay has revolutionised genetic toxicology by providing a reliable and sensitive detection of DNA damage with insight into heterogeneous response of the analysed group of cells.

Based on the majority of study results, it was concluded that resin-based materials (adhesives) are not suitable for covering the pulp because they can cause damage to the pulp cells

(MANTELLINI *et al*., 2003). In addition, composite resin monomers can also be toxic to human gingival fibroblasts (MOHARAMZADEHA *et al.*, 2007).

Nowadays, it is considered that composite materials and adhesives are potentially toxic (GAUTHIER *et al*., 2007), particularly with inappropriate illumination (QUINLAN *et al.*, 2002).

In addition to studies on gingival cells, the primary DNA damage under the influence of composite resins was also studied on human peripheral blood leukocytes. Thus, TADIN *et al*. (2013) performed biomonitoring of flowable and non-flowable composite resins and found limited genotoxic activity in human peripheral blood leukocytes which do not pose a significant risk to human DNA. On the other hand, two resin-based root-canal sealers (Epiphany and RealSeal) and their components were also tested on human leukocytes *in vitro*. (BARABA *et al.*, 2011). Although these sealers induced increase of primary DNA damage after 4 hours of treatment, after 24 h they were primarily cytotoxic and induced apoptosis.

Since there are insufficient literature data about the effects of photopolymerisation time on genotoxicity of adhesives, the aim of this paper was to examine the possible genotoxic effects on non-polymerised, partially polymerised and polymerised adhesives using *in vitro* Comet assay on isolated human peripheral blood lymphocytes.

MATERIALS AND METHODS

Preparation of adhesive solutions and photopolymerisation

Adper Single bond2 and *Adper Easy One/3M ESPE* adhesive photopolymerisation was performed by *Elipar Highlight* 3M ESPE halogen lamp for 0, 10 and 40 seconds. The solutions of adhesives for the examination of genotoxicity were prepared the following way: 1. Three series of solutions were prepared under the same conditions. 2. 1 mL of distilled water was poured in each test tube. 3. 50 μL of non-illuminated adhesive (witout the influence of halogen lamp) was poured in distilled water. 4.50 μL of adhesive was placed on microscopic slide and photopolymerised using halogen light for 10 sec. 5. The discs of polymerised adhesive raisin were placed in 1 mL of distilled water. 6. Illumination of adhesives for 40 sec was done the same manner. 7. The total mass of 50 μL of adhesive was measured at analytical balance. The average mass of 50 μL of adhesive was 0.045 g. 8. In order to dissolve unpolymerised adhesive the solution was left for 2 days. 9. In 1mL of solution containing dissolved and undissolved adhesive (in a form of pellet for 0 sec of treatment, and in the form of a disc after 10 or 40 sec of treatment) 1 mL of 1.8% NaCl was added. The obtained solution had total volume of 2 mL and concentration of 0.9% of NaCl which is the same as in a saline solution. 10. The solutions were transferred in vials and numerated. 11. The pellets and discs were placed on microscopic slide and dried at 60°C for 2 h. 12. After the drying, the remaining mass was measured at an analytical balance.

Isolation of lymphocytes

Heparinised blood samples (4 mL) were obtained by venepuncture from three healthy male donors under 30 years of age. Lymphocytes were isolated from whole blood with Ficoll-Paque medium and centrifuged at 1900 g 15 min. The lymphocytes forming a layer were directly above Ficoll-Paque. The isolated lymphocytes were washed twice in RPMI 1640 medium, each wash was followed by a centrifugation 10 min at 1800 g. Finally, the supernatant was removed as carefully as possible without disturbing the pellet. An aliquot of 1 ml of RPMI 1640 was added and the pellet was resuspended. A manual cell count and an estimate of cell viability were performed using the Trypan blue exclusion test.

The Comet assay

Alkaline version of the single cell gel electrophoresis (Comet) assay was performed on isolated human peripheral blood lymphocytes according to SINGH *et al*. (1988) with slight modifications (TICE *et al*., 2000; SPEIT and ROTHFUSS, 2012). Microscope slides were precoated with 1% normal melting point agarose and allowed to air dry at room temperature for at least 48 h. Suspension of isolated lymphocytes in PBS was treated with adhesives for 30 min at 37°C at final concentrations of 100, 200, 500 and 1000 μg/mL. After incubation with the tested compound the cell viability was evaluated using Trypan blue exclusion test. After centrifugation (5 min at 2000 rpm), 100 μL of cell suspension was mixed with 100 μL of 1% low melting point agarose (LMPA). The 90 μL of suspension was rapidly pippeted onto the first agarose layer and spread using a coverslip, and put in the fridge to solidify. After removal of the coverslip, the 90 μL of 0.5% LMPA was added as the third layer, spread using a coverslip and allowed to solidify at 4°C for 5 min. Afterwards, the slides were immersed in cold lysis solution at pH 10 (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10, 1% Triton X–100, 10% DMSO) overnight at 4°C. After lysis, the slides were placed in a horizontal gel electrophoresis tank to allow DNA unwinding in cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 30 min. Electrophoresis was done at 4°C with electric current of 25 V and 300 mA for 30 min. All these steps were performed under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. The slides were then neutralized with 400 mM Tris-HCl (pH 7.5) for 5 min. The neutralisation was repeated three times. Then, the slides were fixed with cold methanol, dried and stored. Before analysis, the slides were rehydrated with ice cold distilled water and stained with 50 μl of 20 μg/ml ethidium bromide.

RESULTS

The results of the research of adhesive genotoxicity are shown in Fig. 1 to 6. A comparison of differences in the structures of the obtained results was done using χ^2 test. It has been demonstrated that DNA damage (A and E comet class), during the testing of the two adhesives, does not differer statistically in the same treatment (the same duration of polymerisation and the same adhesive concentration). The χ^2 test results of the first adhesive – *Adper Single Bond 2*, with photopolymerisation time 0 to 10 seconds, showed that there was a statistically significant increase of DNA damage, compared to the negative control (solvent). With 10 sec polymerisation time the degree of statistical significance was lower, compared to treatment with adhesive without photopolymerization (polymerization time 0 seconds). However, even at 10 sec. time the *Adper Single Bond 2* was not polymerised completely, so it exhibited genotoxic effects at all experimental concentrations. Contrary to this, after polymerisation with *Adper Sinlge Bond 2* for 40 seconds, with all the tested concentrations of this adhesive, the degree of DNA damage with Comet assay was not statistically different ($P>0.05$) compared to the negative control. Similar results were obtained with *Adper Easy One* adhesive. Significant genotoxic effect was not found only with photopolymerisation time of 40 seconds which is detectable by Comet assay. However, photopolymerisation for 10 and 0 seconds was not enough to prevent genotoxic effect, but DNA damage was more profound with unpolymerised adhesive (i.e. without illumination), compared to photopolymerisation for 10 seconds.

The results of statistical analysis (χ^2 test) of the obtained results for the Adper Single Bond 2 adhesive are presented in Table 7, while the results for the Adper Easy One bond are presented in the Table 8.

*Table 1. Analysis of DNA damage on isolated human lymphocytes treated with solutions of Adper Single Bond 2 adhesive. The adhesive was not illuminated. C- denotes negative control, whereas C+ denotes positive control (100 μM H_2O_2). Unpolymerised adhesives produced highly significant (***P<0.001) increase of DNA damage at all concentrations of the adhesive.*

Treatment	Total (E)	High (D)	Medium (C)	Low (B)	None (A)
C-	0	0.5	1.5	3.5	94.5
100***	1.5	7.5	11	14.5	65.5
200***	3	10.5	7.5	12.5	66.5
500***	2	9	10.5	15.5	63
1000***	4	9	10.5	17	60
C+***	67	13.5	7.5	6	5

*Table 2. Analysis of DNA damage on isolated human lymphocytes treated with Adper Single Bond 2 adhesive. The adhesive was illuminated by halogen light for 10 sec. C- denotes negative control, whereas C+ denotes positive control (100 μM H_2O_2). Polymerisation time of 10 sec. was not enough to prevent DNA damage, so there was significant (*P<0.05; P<0.01;***P<0.001) increase of DNA damage at all concentratrions of the adhesive.*

Treatment	Total (E)	High (D)	Medium (C)	Low (B)	None (A)
C-	0	0.5	1.5	3.5	94.5
100*	1.5	4.5	7.5	10.5	77
200**	2.5	6	8	10	73.5
500**	3	6.5	6.5	15.5	71
1000***	4	8.5	8.5	10.5	68.5
C+***	67	13.5	7.5	6	5

*Table 3. Analysis of DNA damage on isolated human lymphocytes treated with Adper Single Bond 2 adhesive. The adhesive was illuminated by halogen light for 40 sec. C- denotes negative control, whereas C+ denotes positive control (100 μM H_2O_2). Polymerisation time of 40 sec. prevented DNA damage. Only the positive control gave rise to a significant (***P<0.001) DNA damage.*

Treatment	Total (E)	High (D)	Medium (C)	Low (B)	None (A)
C-	0	0.5	1.5	3.5	94.5
100	0	0.5	5	5	88.5
200	0.5	2	3.5	6.5	87.5
500	1	1.5	2.5	6	89
1000	0	2.5	3.5	6	88
C+***	67	13.5	7.5	6	5

*Table 4. Analysis of DNA damage on isolated human lymphocytes treated with Adper Easy One adhesive. The adhesive was not illuminated. C- denotes negative control, whereas C+ denotes positive control (100 μM H_2O_2). Unpolymerised adhesives produced highly significant (***P<0.001) increase of DNA damage at all concentrations of the adhesive.*

Treatment	Total (E)	High (D)	Medium (C)	Low (B)	None (A)
C-	0	0	2	4.5	93.5
100***	1.5	5.5	6	11	76
200***	2	5	8	11	74
500***	2.5	5	8	13	71
1000***	2.5	5.5	9	13.5	69.5
C+***	67.5	11.5	9	7.5	5.5

*Table 5. Analysis of DNA damage on isolated human lymphocytes treated with Adper Easy One adhesive. The adhesive was illuminated by halogen light for 10 sec. C- denotes negative control, whereas C+ denotes positive control (100 μM H_2O_2). Polymerisation time of 10 sec. was not enough to prevent DNA damage, so there was a significant (*P<0.05; P<0.01;***P<0.001) increase of DNA damage at all concentratrions of the adhesive.*

Treatment	Total (E)	High (D)	Medium (C)	Low (B)	None (A)
C-	0	0	2	4.5	93.5
100*	1	4	5.5	11	79
200**	2	5	5	8	77
500**	1	5.5	6.5	10.5	76.5
1000***	2	5.5	6	9.5	74.5
C+***	67.5	11.5	9	7.5	5.5

*Table 6. Analysis of DNA damage on isolated human lymphocytes treated with Adper Easy One adhesive. The adhesive was illuminated by halogen light for 40 sec. C- denotes negative control, whereas C+ denotes positive control (100 μM H_2O_2). Polymerisation time of 40 sec. prevented DNA damage.Only the positive control gave rise to a significant (***P<0.001) DNA damage.*

Treatment	Total (E)	High (D)	Medium (C)	Low (B)	None (A)
C-	0	0	2	4.5	93.5
100	0	0.5	3	6	90.5
200	0	0	4.5	5	90.5
500	0.5	0.5	2	8	89
1000	0	0.5	4.5	6	89
C+***	67.5	11.5	9	7.5	5.5

Table7. Statistical analysis of the values of various DNA damages at different times of illumination and various concentrations of Adper Single Bond2 adhesive in comparison to the controls

Treatemet		Negative control		Positive control	
Polymerisation time (sec)	Concentrations	χ^2	*p*	χ^2	*p*
0	1000	32,330	<0,001***	103,100	<0,001***
	500	28,000	<0,001***	108,385	<0,001***
	200	25,202	<0,001***	109,503	<0,001***
	100	24,550	<0,001***	114,426	<0,001***
10	1000	21,232	<0,001***	108,678	<0,001***
	500	18,403	0,001**	115,494	<0,001***
	200	15,795	0,003**	119,259	<0,001***
	100	12,629	0,013*	125,688	<0,001***
40	1000	8,534	0,074	130,785	<0,001***
	500	4,885	0,299	138,281	<0,001***
	200	3,691	0,449	143,167	<0,001***
	100	2,101+	0,552	148,533	<0,001***

For χ^2 -without a sign + number of degrees of freedom is $\nu = 4$, with sign + $\nu = 3$

Table 8. Statistical analysis of the values of various DNA damages at different times of illumination and various concentrations of Adper Easy One adhesive in comparison to the controls

Treatemet		Negative control		Positive control	
Polymerisation time (sec)	Concentrations	χ^2	*p*	χ^2	*p*
0	1000	31.839	<0,001***	104.631	<0,001***
	500	27.636	<0,001***	110.001	<0,001***
	200	24.963	<0,001***	111.226	<0,001***
	100	24.151	<0,001***	115.812	<0,001***
10	1000	21.318	<0,001***	110.431	<0,001***
	500	18.242	0,001**	116.773	<0,001***
	200	15.713	0,003**	120.501	<0,001***
	100	12.546	0,014*	126.794	<0,001***
40	1000	8.613	0,072	131.800	<0,001***
	500	5.156	0,272	139.322	<0,001***
	200	3.794	0,435	144.069	<0,001***
	100	2.039	0,729	83.261	<0,001***

Number of the degrees of freedom $\nu = 4$

DISCUSSION

The biological safety of new materials used in dentistry practice represents a very important issue in evaluation of their side-effects. Since the dentin bonding agents come into contact with surrounding tissues, it is very important to evaluate any possible toxic and genotoxic effects. As for the genotoxicological analysis, each material has to be evaluated in several *in vitro*

and *in vivo* tests from the list made by "Organisation for Economic Co-operation and Development (OECD)". The Comet assay is one of the tests recommended by OECD.

This study conducted research of genotoxic effect of composite adhesives (bonds) which are nowadays commonly used in dental practice (adhesives of the latest generation). One adhesive (*Adper Sinlge Bond 2*) belongs to the group used after etching and rinsing of dental tissue, whereas the other (*Adper Easy One)* belongs to self-etch adhesives. Since they contain potentially toxic monomers, the evaluation of the genotoxic effects of these adhesives, with respect to the length of illumination, i.e. photopolymerisation, would contribute to a better understanding of the possible biological changes of the pulp, caused by their reaction.

Considering the fact that there is not enough information about the effects of different types of photopolymerisation on genotoxicity of composite adhesives, the application of the Comet assay for the currently used adhesives is appropriate. In addition to non-polymerised adhesives (illumination for 0 seconds), two time limits were also used, namely, photopolymerisation for 10 and 40 seconds. The results showed that neither of the two adhesives demonstrated statistically significant genotoxic effects with photopolymerisation for 40 seconds. Therefore, this photopolymerisation time could be recommended as relatively safe, although the time limit at 10 seconds, which is most frequently used in dental practice, is shown by our research to be of insufficient length to completely neutralize the potentially genotoxic effects of both adhesives.

Numerous studies have shown that in clinical conditions, the treatment of the pulp with adhesives can lead to continuous release of non-reacted adhesive components and pulpal inflammation. It has been proved that composite adhesives are cytotoxic to odontoblast-like cells. When adhesive material was illuminated, the cytotoxic effect decreased. This has been confirmed, among others, by research conducted by MANTELLINI *et al*. (2003). Odontoblast-like cells of the mouse (MDPC-23), non-differentiated pulpal cells (OD-21) or macrophages (RAW 264.7) were exposed to *Single Bond*, polymerized for 0-40 seconds. A significant difference was observed in cell response to the three conditions of photopolymerisation (illumination for 0, 10 or 40 seconds). Non-polymerised and partially polymerised adhesive resin caused a sudden apoptosis in all cells (MANTELLINI *et al.*, 2003).

Our research also confirmed toxic effect of non-polymerised and partially polymerised adhesives (for 0 and 10 seconds illumination of adhesives there was a genotoxic effect in lymphocytes of peripheral circulation in humans).

On the other hand, some *in vivo* studies have shown that composite resins, including composite adhesives, are biocompatible with the dental pulp (COSTA *et al.*, 2003). According to these studies, the application of composite adhesives directly on animal pulp does not cause pulp necrosis, which was determined to be a consequence of the remaining bacteria and their toxins.

The research by KNEŽEVIĆ *et al*., (2008) determined the difference in toxicity between composite materials illuminated in various ways by LED device, using the Comet assay. Significant cytotoxicity was discovered in non-polymerised composites and in composites polymerised by HIP polymerization method. Illumination by low light intensity (LOP) program showed the lowest toxicity (KNEŽEVIĆ *et al.*, 2008). Therefore, it was concluded that longer illumination of composites using low intensity light leads to lower toxicity, compared to shorter exposure to illumination.

Comet assay was also used in this research and it was confirmed that longer illumination (40 seconds) causes lower genotoxicity, not only of restorative composite materials but also of composite adhesives.

CONCLUSIONS

Photopolymerisation for 0 and 10 seconds did not prevent genotoxic effects which are more significant with photopolymerisation for 0 seconds (i.e. when adhesives are not illuminated), compared to photopolymerization for 10 seconds. Comet assay did not detect any significant genotoxic effects with photopolymerisation for 40 seconds. Based on the results, it can be concluded that polymerisation for 10 seconds is insufficient to neutralise the genotoxic potential of the two tested adhesives.

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UTICAJ FOTOPOLIMERIZACIJE NA GENOTOKSIČNOST KOMPOZITNIH ADHEZIVA U KOMET TESTU

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Izvod

Pojedine *in vivo* studije pokazale su da primena atheziva direktno na otvorenu pulpu ili na tanka sloj dentina dovodi do upale i apscesa pulpe. Ova reakcija dovodi se u vezu sa toksičnim efektima monomera oslobođenih iz adheziva. Pokazano je da adekvato osvetljavanje čini adhezive manje toksičnim. Cilj ovog rada bio je da se ispita genotoksičnost nepolimerizovanih, delimično polimerizovanih i polimerizovanih adheziva na izolovane limfocite čoveka primenom alkalne verzije komet testa. *Adper Single bond2* i *Adper Easy One/3M ESPE* adhezivi su fotopolimerizovani pomoću *Elipar Highlight* 3M ESPE halogene lampe u trajanju 0, 10 i 40 sec, pri njihovim finalnim koncentracijama od 100, 200, 500 and 1000 μg/mL. Oba adheziva pri fotopolimerizaciji od 0 i 10 sec. dovode do značajnog porasta oštećenja DNK u odnosu na vrednosti negativne kontole (rastvarač). S druge strane, nakon fotopolimerizacije u trajanju od 40 sec. oba adheziva pri svim ispitivanim koncentracijama ne dovode do značajnih razlika ($P>0.05$, χ^2 test) u stepenu oštećenja DNK u komet testu u poređenju sa vrednostima negativne kontrole. Prema tome, samo je vreme fotopolimerizacije od 40 sec. bilo dovoljno da spreči genotoksične efekte oba adheziva u komet testu.

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