UNIVERSITY OF LATVIA
FACULTY OF MEDICINE

GENOTOXICITY OF BISPHENOL A CONTAINING DENTAL COMPOSITES

DIPLOMA THESIS

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ABSTRACT

Some dental resin composites contain BPA-based monomers elucidating genotoxicity to living cells and an ability to cause living cell death. These monomers have an ability to degrade under the action of esterases present in human saliva, therefore releasing toxic degradation products into the oral environment. Released monomers can even penetrate structures of the tooth and enter the bloodstream through the pulp. Aims of this study were to investigate the effect of dental composite materials on the cells of human bloodstream, particularly on the lymphocytes, and to evaluate damage levels of the cell DNA. To study this question 4 different commercially available dental composite fragments were soaked in isolated human peripheral blood mononuclear cells from whole blood samples of 10 healthy subjects and analysed by the means of alkaline comet assay. Obtained results have shown an increased level of DNA single-strand breaks after 24h incubation of each sample containing a dental composite. Regarding the observed dental resin composite genotoxic effect on human lymphocytes, dental practitioner attention should be emphasized to the proper composite polymerization during setting procedure and accurate post-polymerization adjustment and polishing.

Further investigation is required to evaluate different exposure times, however current results show that 20 second exposure time may not be enough for a complete polymerization of a composite. Additional studies are also required to test other commercially available composites to evaluate their overall genotoxicity and find the safest option for practical uses.

ABBREVIATIONS

APN – Apoptic nuclei

AU - Arbitrary units

bis-DMA – Bisphenol A dimethacrylate

bis-EDMA – Bisphenol A ethoxylate dimethacrylate

bis-EMA – Bisphenol A ethoxy methacrylate

bis-GMA – Bisphenol A2 glycidyl methacrylate

bisHPPP – 2,2-Bis[4phenyl]propane

BPA – Bisphenol A

BW – Body weight

DC – Degree of conversion

D - Damage index

DMSO - Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

DNA-DSBs - DNA double-strand breaks

EFSA - European Food Safety Authority

ELISA – Enzyme-Linked Immunosorbent Assay

EMPA - 2,3-epoxy-2-methylpropionic acid

EMPME – 2,3-epoxy-2-methyl-propionicacid-methylester

ESI – Electrospray Ionization

FBS – Fetal Bovine Serum

GC – Gas Chromatography

HEMA - 2-hydroxyethylmethacrylate

IQR - Interquartile range

LC – Liquid Chromatography

LD – Lethal dose

LED – Light Emitting Diode

MS – Mass Spectrometry

NMDA – N-methyl-D-aspartate

NOAEL – No observed adverse effect level

PBMNC - Peripheral blood mononuclear cells

PBS – Phosphate Buffer Saline

PC – Polycarbonates

SCGE – Single Cell Gel Electrophoresis

SD – Standard deviation

TDI – Tolerable daily intake

TEG-DVBA – Triethylene glycol-divinylbenzyl ether

TEGDMA – Triethylene glycol dimethacrylate

UDMA – 1,6-bis-(methacryloyloxy-2-ethoxycarbonylamino)-2,4,4-trimethylhexane

UV – Ultra Violet

INTRODUCTION

Bisphenol A is released by many resin components, including restorative materials, pit and fissure sealants, and resins aiming to seal orthodontic appliances. In vitro and in vivo adverse effects have been noted and the severity of these effects has been evaluated. A few toxic, genotoxic, and allergic reactions have been shown, displaying minor to severe responses. Many health concerns are documented, with increasing severity. During rodent development, cardiovascular, brain, and developmental deficiencies; obesity; and adverse effects of BPA (Bisphenol A) have been well documented. In adult animals, as well as in humans, severe pathologies have been identified, such as diabetes, defective male and female genital tracts, ovarian cysts, and/or precancerous and cancerous lesions. However, it is difficult to extrapolate from animal pathologies to human. Therefore, the question of the potential adverse effects of resins releasing BPA remains open (Vandenberg et al., 2012).

Although some answers deny any adverse effects on public health in view of the small quantities released by BPA from restorative resins or sealants, the level being below the "non-detectable adverse impact level", some problems raise new insights and result in a revaluation of the protection of BPA in medicine. For a long period of time, the dose level was the most important point. Traditional classical dogma in toxicology was "the dose makes the poison". Evolution of the concept suggests that effects may be detected with low dose. The effects of low doses cannot predict the effects observed at higher doses (Vandenberg et al., 2012).

This implies that the effects of low doses must be taken into consideration in terms of undesirable effects and of possible induced pathologies, doses below that used for traditional toxicological studies (Moon et al., 2012).

The majority of dental composites unleash TEGDMA (triethylene glycol dimethacrylate) *in vitro* and *in vivo*. This component is toxic. The compound induces allergies and cytotoxicity. Many reports describe allergic dermatitis in dental personnel, but far less in the oral cavity of patients. In an estrogen-sensitive cell line, estrogenic effects were found with BPA, bis-DMA (Bisphenol A dimethacrylate), and bis-GMA (Bisphenol A2 glycidyl methacrylate), but not with TEGDMA. Monomers cause adverse biological effects in mammalian cells. TEGDMA causes gene mutations *in vitro*. The formation of micronuclei indicates chromosomal damages, and monomers such as TEGDMA and HEMA (2-hydroxyethylmethacrylate) induce DNA (deoxyribonucleic acid) strand breaks. The comet assay is used to quantify the DNA single-strand breaks, alkali labile, and incomplete excision repair sites (Kleinsasser et al., 2004).

Purpose of the project

- To investigate the effect of dental composites on the human blood cells
- To evaluate and compare levels of DNA damage caused by commercially available dental composites

LITERATURE REVIEW

General description of dental composites

Dental resin composites are frequently used in practical dentistry to rebuild defective or teeth affected by caries (Chan et al., 2010). Caries is a common disease in humans, causing painful sensations and loss of teeth due to the active demineralization of tooth structure and further destruction (Selwitz et al., 2007). Furthermore, dental composites are used to improve teeth aesthetics. Dental composites are developed to replace amalgam material which is used as restorative material but is of a higher toxicity and different aesthetic applications. Amalgam use in practice is already prohibited in several countries, for example, in Scandinavian countries (Chan et al., 2010). Treatment material and composite substitute of choice could be amalgam, gold or glass ionomer filling material (Hes et al., 1999; Knosp et al., 2003). Dental composites have low toxicity to the living cells accompanied by aesthetic and mechanical properties that are suitable for restoration procedures (Ferracane, 2011; Marigo et al., 2015). Main features of composites are satisfying translucency, adequate high resistance to compression force, low wear from friction and easy handling (COUNCIL ON SCIENTIFIC AFFAIRS, 2003; Hegde et al., 2011). Major properties of a composite, such as an ability to withstand mechanical stress and dentine-like colour tone, are determined by the presence of the Bisphenol A based methacrylate cross-linking monomers, for example, bis-GMA and TEGDMA (Bowen, 1964, 1963; Cramer et al., 2011; Van Landuyt et al., 2011). Various quantities of these monomers can be found depending on product type (Peutzfeldt, 1997). This material should be handled with specialized instruments when it is in the form of soft and viscose plaster, placed on desired prepared cavity, or if flowable composite – injected into the cavity, and light cured with visible blue light (Chan et al., 2010; Ferracane, 2011; Petrović et al., 2015).

Dental composite degree of conversion (DC) is a polymerization process, forming polymer chain by converting double carbon bonds into single bonds. To completely transform monomers to a more complex polymer additional polymerization is necessary. Polymerization starts with the absorption of light in 400 to 500 nm wavelength range, as the result aliphatic amine reacts, producing free radicals (Acquaviva et al., 2009; Noronha et al., 2010). Not all monomers are changing their structure completely, for example, dimethacrylate polymer remains in a state of unsaturated monomer (Costa et al., 2011; Galvão et al., 2010; Ozturk et al., 2013; RIBEIRO et al., 2012). While evaluating DC, one must consider light source, size of the light tip, light tip contact with curing material, power density, wavelength, irradiation time, structure of the organic matrix, dispersion and amount of inorganic fillers, cured composite thickness and shade of the composite (Cekic-Nagas et al., 2011; Galvão et al., 2010; Yoshikawa et al., 2013).

Chemical properties of dental composites

One of the most popular ways to investigate dental composite material is to test variation of mechanical properties – wear, softening, stress or fatigue fracture. Chemical degradation (biodegradation) accelerates these processes (Santerre et al., 2001). The process of degradation in water environment is catalysed by the presence of saliva and bacterial esterases. The higher percentage of esterases present in saliva, the faster is the degradation process (Bourbia et al., 2013; Finer and Santerre, 2004a). In bis-GMA this process breaks single bonds between the acyl group and the unprotected oxygen in ester group. In the result a gap occurs, where degradation products like BPA can be released (Göpferich, 1996). In case of UDMA (1,6-bis-(methacryloyloxy-2-ethoxycarbonylamino)-2,4,4-trimethylhexane) – urethane bonds have an ability to decrease the degradation rate of ester-bonds (Finer and Santerre, 2003; Xu et al., 2017).

Studies have shown that enzymes in the saliva can degrade composite bis-GMA and TEGDMA monomers within one day (Jaffer et al., 2002).

Esterases in the human saliva affect composite restorations on their surfaces – they are too large to enter polymer network and transport deeper in the restoration. With time esterases tends to degrade more and more material due to the wearing processes of a composite (Finer and Santerre, 2004b).

Methanol is an alcohol that is aggressive to a bis-GMA containing resins and has the ability to degrade them (Hope et al., 2016; PubChem, n.d.). Similarly, 50–75% ethanol is capable to destroy polymer structure, but is weaker than methanol, resulting in the composite to expand and becoming more porous (Ferracane, 2006; PubChem, n.d.; Wu and McKinney, 1982). Both alcohols can extract phenolic (also antioxidant) compounds from phenol containing products. Acetone is a similar solvent, but weaker in comparison with two alcohols, and water is the weakest amongst them all (Boeing et al., 2014).

Surface characteristics of a composite play a crucial role in the release of the unbound monomers. Smoothly polished surface of a polymerized composite decreases the release of monomers. Unpolished cured composite surface has oxygen inhibition layer coating, that contains more of these monomers than the polished counterpart (Wataha et al., 1999). Oxygen inhibition layer is created at the moment when composite is light-cured in the presence of air. Presence of the oxygen in the air makes the free-radical polymerization slower (Bijelic-Donova et al., 2014). Oxygen itself is a strong inhibitor that reacts with free radicals forming a non-reactive peroxy radicals, that tends to form an inactive product of its reactions (“Radical Chain Polymerization,” 2004). Viscosity of dental composites impact thickness of the oxygen inhibition layer. Composites with lower viscosity has thicker oxygen inhibition layer (Nunes et

al., 2006). Thickness of the oxygen inhibition layer furthermore could be affected by properties of composites - monomer chemical structure, free radical amount and concentration, filler form and temperature, the rate of oxygen usage (Bijelic-Donova et al., 2014).

The same monomers containing composites are also used in orthodontics for fixation, for example, brackets (Çörekçi et al., 2014; Hope et al., 2016). Composite residual monomers, bis-GMA and TEGDMA, after polymerization process are cytotoxic, and the toxicity is even higher if composites are insufficiently polymerized (Çörekçi et al., 2014). Cytotoxicity is the lethal or sublethal consequence of the application of cytotoxic agents. In case of lethal result affected living cells die and if sublethal – cell proliferation or growing/dividing activity is decreased ("Cytotoxicity - LV," n.d.). These toxic monomers can penetrate dentine, pulp or mucosa after composite polymerization with local or systemic effect (Geurtsen, 1998; Schmalz, 2009). Monomers from composites used in orthodontic braces for fixation even has the ability to penetrate enamel (Çörekçi et al., 2014).

There are few studies investigating every component of dental composites, particularly monomers for genotoxicity and cytotoxicity and ability to cause DNA double-strand breaks (DNA-DSBs) (Yang et al., 2018). Released monomers also showed mutagenicity and teratogenicity (Schwengberg et al., 2005). Genotoxicity arises from damage to the living cells DNA (genetic material), altering changes in cell DNA original function and proliferation cycle ("Genotoxicity - LV," n.d.). In human oral mucosa fibroblasts can form another compound, that is extremely toxic to DNA - 2,3-epoxy-2-methyl-propionicacid-methylester (EMPME) from 2,3-epoxy-2-methylpropionic acid (EMPA), that is TEGDMA and HEMA metabolite (Mahaney et al., 2009; Reichl et al., 2010; Schwengberg et al., 2005; Seiss et al., 2007).

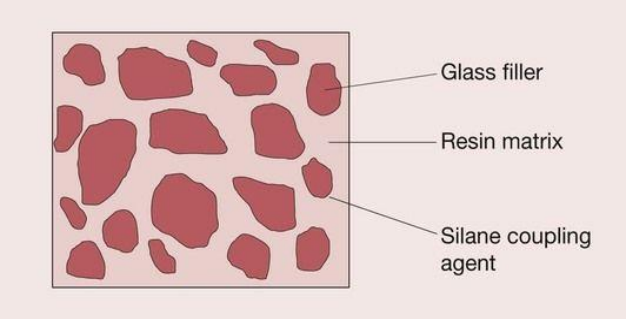

Figure 1. Components of dental composites (Themes, 2015).

In the presence of saliva toxic effects are reduced, because monomers bind to larger molecules (proteins) (Rothmund et al., 2015; Tsitrou et al., 2014). All dental composites share the structure and have three main components (Figure 1); one is a non-organic filler (50-70% of total volume), second is organic and soft resin polymer matrix, third is a coupling agent – silane, that tolerate mechanical stress distribution (Finer and Santerre, 2007).

Structural features of dental composites

Dental composites have high density due to few free spaces between different structural elements. Composites with dense structural organization tend to have lower degradation rate during wearing process (Arima et al., 1996). Fillers can have different morphology – form of fibers, sheets or particles (Alsharif et al., 2010). Novel dental composite filler particles are made from silicone oxide, barium, zirconium, ytterbium (add ability to release fluoride), strontium (Lyapina et al., 2016; Mystkowska, 2009; Polydorou, 2018). Polymer matrix is made up of monomers, stabilizers, pigments, an initiator-activator structure and photoinitiators (Durner et al., 2010; Marghalani, 2014). Monomers in the matrix could be bis-GMA with TEGDMA (always together because TEGDMA dilutes highly viscous bis-GMA), UDMA, bis-EMA (Bisphenol A ethoxy methacrylate), bis-EDMA (Bisphenol A ethoxylate dimethacrylate), bis-DMA (Salehi et al., 2015; Sarrett, 2015; Sideridou and Achilias, 2005; Xu et al., 2017). The use of UDMA based composites are becoming wider than its bis-GMA counterparts as a consequence that UDMA containing composites have more favourable characteristics such as higher filler percentage, lower viscosity and superior toughness (Abed et al., 2015; Kerby et al., 2009; Matsukawa et al., 1994; Sideridou et al., 2002; Xu et al., 2017). There are several ways to dissolve a composite - with mechanical wearing, contact with saliva, food, drinks, substances, drugs, bacteria (Bourbia et al., 2013; Finer and Santerre, 2004b, 2003; Øilo, 1992). UDMA in comparison to bis-GMA based composites in the human oral environment are less soluble under action of the enzymes – esterases (Park et al., 2009).

Some studies suggest that novel dental composite components UDMA/TEG-DVBA (triethylene glycol-divinylbenzyl ether), could be promising substitutes of bis-GMA/TEGDMA because of their superior mechanical properties, different polymerization mechanism and minimized release of non-reacted monomers (Wang et al., 2018).

Description of BPA

Bisphenol A containing monomer in dentistry was introduced in 1962 by Dr. L.Bowen and it was bis-GMA (Krishna Ravi et al., 2013). BPA is a popular chemical widely used in manufacturing of plastic material, thermal paper and epoxy resins due to its ability to improve different major properties of a material (Geens et al., 2011; Goodman and Peterson, 2014; Wells, 2019). It dissolves in any biological solvent, and incompletely biodegrades in water. At the room temperature it is white solid flake or crystal (Zielińska et al., 2019).

It was synthesized in 1891, but first studies were conducted in 1930, when there was a need for synthetic estrogen (Wells, 2019). In 1940 first Bisphenol A containing plastic was produced (Zielińska et al., 2019). In paper manufacturing it is used in a free form and can be easily absorbed into bloodstream through the skin (Biedermann et al., 2010; Hormann et al.,

2014; Kaddar et al., 2008). Huge amounts of BPA are produced every year, and more than 100 tons are discharged into the air (Rubin, 2011). Several soil and wastewater-living bacteria, fungi and algae are capable to degrade Bisphenol A. BPA solubility in water is 200 mg/L at 25 °C. It is enough to cause adverse effects to occur. Immune response of a living organism can be seen starting with 1 x 10^{-5} g/L (Zielińska et al., 2019).

About 90% of Bisphenol A are used in the production of other chemicals and small quantities of it can still be found in the final product. It has low evaporation rate, quickly degrades when released into the atmosphere (Goodman and Peterson, 2014).

Bisphenol A in manufactured products exists in two forms: free form or when it is modified - polymerized or conjugated (Goodman and Peterson, 2014; Vandenberg et al., 2009). Epoxy resins and polycarbonates (PC) are most popular derivates. The most broadly used products - epoxy resins, are formed from reaction of epichlorhydrine with water (Wells, 2019). BPA in epoxy resins is considered to be stable and is released only as a leftover chemical (Zielińska et al., 2019).

People are consuming BPA with food that was in contact with polycarbonate plastics or epoxy resins from storage containers (also from metallic can lining). Exposure to BPA is calculated from urine and takes into account processes of absorption, distribution, metabolism. And the final excretion of BPA from human organism is from <0.03 to 1.61 μkg^{-1} day^{-1}. But elimination from food is maximum <0.01 – 13 $\mu kg^{-1}day^{-1}$. Higher amounts of chemical are estimated to be in urine and lower in the blood (Goodman and Peterson, 2014). It is possible to locate detectable levels of BPA also in human sweat (Genuis et al., 2012). Some studies show that it takes 5 – 7 hours for the high dose of BPA to be completely removed from the organism.

Metabolism of this chemical mainly occurs in the liver and intestine (Figure 2), and in the end segregates with the urine in the form of glucuronide. BPA excreting speed with urine is low: about 2–3 $\mu g\ l^{-1}$, can even be mistaken as statistical noise.

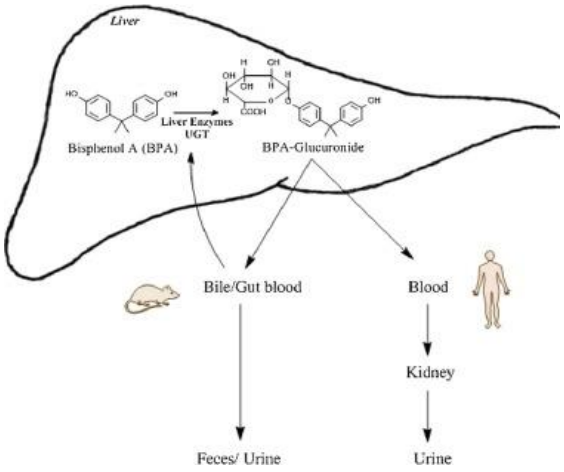

Figure 2. A metabolic pathway of BPA (Jalal et al., 2018).

After the metabolic pathway BPA has ability to repeat circulation through intestine and liver thereby extending the amount and time of it in blood circulation (maximum 1% from intake dose) (Goodman and Peterson, 2014). There is some evidence from studies showing that BPA is present in the urine of more than 90% of world population (Wells, 2019).

European Food Safety Authority (EFSA) in January 2015 lowered tolerable dietary daily intake of BPA to a value of 4 μg/kg body weight (BW)/day, when first side effect might occur. Exceeding this dose inevitably lead to changes in reproductive organs, metabolism, liver, kidneys, neurons and/or immune system. In 2007 the daily intake limit was higher - 50 μg/kg body weight (BW)/day. Higher daily intake limit was accepted due to the lack of animal studies. Additional studies have shown that human beings have an elevated level of BPA in their organism due to the everyday contact with BPA - containing materials, especially infants and toddlers have high chances to receive BPA from canned food and bottles. Average daily intake of BPA for adults is 145 ng/kg BW/day (EFSA Panel on Food Contact Materials, 2015).

Due to the lipophilic nature of BPA its accumulation rate in adipose tissues is three times higher than in any other tissue (Genuis et al., 2012).

Bisphenol A may be released for a short time (about several hours) after setting of composite or tooth fissure sealants, that are used to treat or prevent occurrence of caries lesions in teeth (Goldberg, 2014).

Toxicity of Bisphenol A and its derivatives

To adjust the properties of a composite resin, a similar chemical is sometimes added - bis-DMA, it dissolves in the saliva at a relatively low rate, and releases BPA like bis-GMA ("ADA-Statement-on-Bisphenol-A.pdf," n.d.). Bis-DMA mimics lesser estrogen functions in comparison with other Bisphenol A based monomers. Every monomer where Bisphenol A is a base material, from which further chemicals are made, has leftovers of BPA ("ADA-Statement-on-Bisphenol-A.pdf," n.d.; Goldberg, 2014).

Bis-GMA under the effect of high temperature (100 °C during 3 h) has an ability to transform into components, that have a 26.3% of BPA content (Deviot et al., 2018).

Bisphenol A and bis-GMA are the most toxic monomers in dental composites for the living cells. There is an increase in toxicity after hydrolysis followed by production of methacrylic acid, that influences cell permeability, present estrogenicity and can be detected in dental pulp/cement and oral mucosa (Lefebvre et al., 1996). Methacrylic acid have destructive activity to the tissues and metals ("METHACRYLIC ACID | CAMEO Chemicals | NOAA," n.d.). Living cells after contact with low concentration of Bis-GMA are capable to restore activity, but high concentrations are fatal for them (YANO et al., 2011). BPA toxicity is defined by its binding ability to the minor groove of DNA helix (Zielińska et al., 2019).

In contact with living organism with dose starting from 10 nM, begins rapid increase of calcium ion transport through neuron NMDA (N-methyl-D-aspartate) channel. This results in the beginning of minimal changes in the organism (Tanabe et al., 2006). When the intake dose exceeds 39 nM Bisphenol A may inhibit the expression of proliferation markers of human gingival keratinocytes (Ehrenmann et al., 2017). Animal studies of exposures to BPA depending on the dose have shown that the adverse effects at low and high levels are different (Goodman and Peterson, 2014).

BPA has an ability to affect human hormones - estrogens (acting like them and provoking cancer), androgens and thyroid hormones (decreasing due to the antagonistic activity), additionally it may affect other parts of the organism such as cardiovascular and reproductive system, weight and children neural development disturbances (more in prenatal period) (Delfosse et al., 2014; Wells, 2019).

TEGDMA is a monomer with mutagenic activity (because of the ability to covalently bind DNA) and immunosuppressive properties (weakens immune system) (Emmler et al., 2008; Salehi et al., 2015). Mutagenic activities are represented by mutagens (chemical compounds), that induce permanent inheritable modifications in the genetic information. These modifications depend on several factors, including affected region of the DNA sequence. It could cause apoptosis of the affected cells if active proliferating cells are influenced (Schrader, 2003). DNA is a molecule located in all living organisms, containing individual genetic information (Rettner et al., n.d.). UDMA causes lymphocyte DNA damage and consequently leads to their apoptosis (Poplawski et al., 2010). Release of TEGDMA from it containing composites is decreased when light-curing is performed with increased light irradiation time, not less than 40 s and with distance less than 10 mm. When light-curing time is longer, only BPA is released due to the increased temperature, but UDMA and TEGDMA have an opposite course of action and need shorter distance to the light source (Kwon et al., 2015).

Dental adhesive is a liquid material, that is used in dentistry along with resin composites. Adhesive is implemented to the previously prepared cavity, light-cured and then composite material is applied. Adhesive can leak to oral environment during composite setting stages, so its setting must be performed with precision (MacAulay et al., 2017). It also releases another BPA derivative – bisHPPP (2,2-bis[4phenyl]propane), and enzymes esterases may increase the release 1.8 times (Shokati et al., 2010).

Methods of investigation. Comet assay

Methods used to evaluate levels of BPA are different. For example, ELISA (enzyme-linked immunosorbent assay), where one of the BPA characteristics is used – its ability to binds to a larger carrier-like protein (albumin in blood) causing immune response to occur (Goda et al., 2000). Other methods of investigation include liquid chromatography (LC) with UV light detection, electrospray ionization (ESI) with MS (mass spectrometry) detection, gas chromatography/mass spectrometry (GC/MS), liquid chromatography with mass spectrometry detection (Gallart-Ayala et al., 2007; Manabe et al., 2000; Mazzaoui et al., 2002; Watabe et al., 2004).

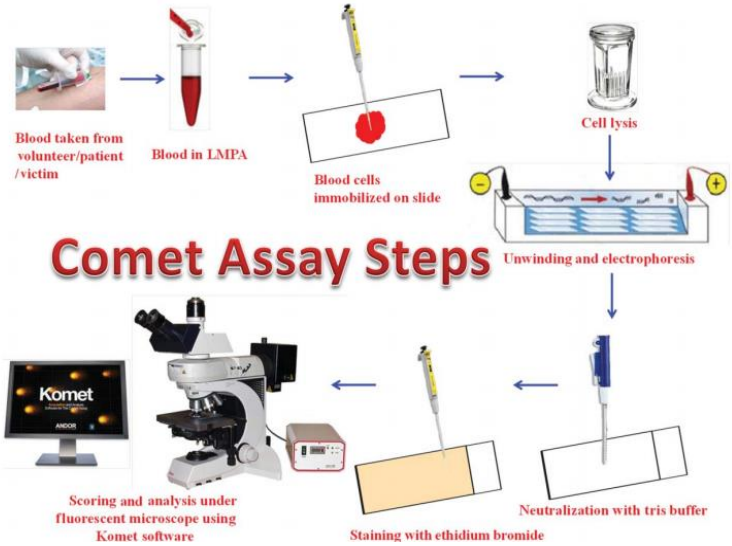

Figure 3. A schematic representation of the comet assay (Shukla, 2017).

Comet assay or alkaline single cell gel electrophoresis assay (SCGE) is a method which is used to evaluate and detect damage (genotoxicity) to the genetic material – DNA of the living organism cells, for example lymphocytes in blood sample. The most common application of this technique is to evaluate single-stranded DNA breaks. With some alterations to this method it is also possible to measure DNA double-strand breaks, base damage, cross-links, apoptotic nuclei. This method is very sensitive to DNA changes and needs to be tested only on vital blood cells, to avoid mixing the results with apoptotic cells (Kleinsasser et al., 2004; Olive and Banáth, 2006). With fluorescence microscopy after the gel electrophoresis it is possible to visualize cells, fixed in agarose gel, and observe cells with their tails (Figure 3).

These tails are broken and relaxed DNA strains, migrated to the anode direction during the process of electrophoresis (L. Olive et al., 1990). Length of these tails depends on number of DNA single (in case of alkaline comet assay) or double (in case of neutral comet assay) strand breaks. The longer the tail the higher is the DNA damage (Valencia et al., 2011). Tails could also be evaluated visually depending on the fluorescence intensity and/or tail moment. More intensive fluorescence means there are larger numbers of DNA strand breaks. Tail moment could be evaluated from tail length and intensity visualized together. Classification of the results of comet assay method is based on analysing comet tail intensity magnitude. The assessment of a single sample usually involves 100 counted cells. These cells need to be divided into 5 groups – 5 approximate levels of tail intensity as shown on Figure 4 (Collins et al., 1997).

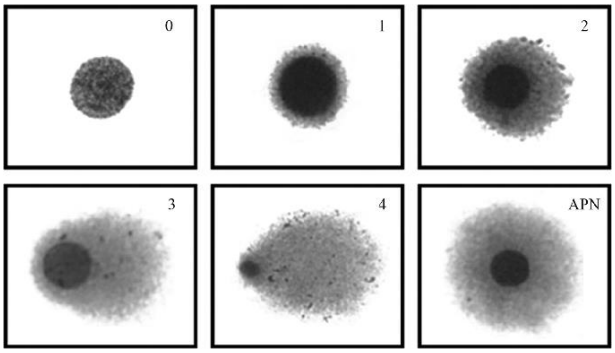

Figure 4. Four types of DNA damage. 0 – healthy cell (no damage), 1 to 4 increased level of DNA damage; APN – apoptic nuclei (dead cell) (Valencia et al., 2011).

Specialized computer software can be used for counting these cells or in other words "comets" (L. Olive et al., 1990). It is possible to easily recognize these apoptic cells whereas DNA fragmentation is extensive (Olive et al., 1993). After gel electrophoresis under alkaline conditions in cell culture storage DNA breaks into single-strands therefore rapidly migrating from the cell forming the "tail"(Valencia et al., 2011). When storage conditions are neutral, no migration can be observed (Olive, 1999). Comet assay allows the evaluation of DNA content and damage in any phase of the cell replication cycle (Olive and Banáth, 2006). In understanding comet assay results it is necessary to keep in mind that DNA damage can be caused by a specific chemical and the biological consequences of that damage (Olive and Johnston, 1997). Also, damage to the mitochondria or cell membrane can be followed by considerable DNA fragmentation with subsequent apoptosis and effect of genotoxicity (Olive and Banáth, 2006). Single-strand breaks detection could be complicated since their detection is blocked by chemicals producing interstrand cross-links (Merk and Speit, 1999).

MATERIALS AND METHODS

(adapted from (Borisovs et al., 2018))

Patients

Permission of the Central Medical Ethics Committee of the Republic of Latvia No 1/17-10-10 was received to perform the study. Informed consent was received from every participant of the study. The comet assay group consisted of 10 patients: 4 males and 6 females of 25 to 67 years (average age of the group: 39.5 ± 1.9 years).

Collection and processing of blood samples

Blood was obtained by vein puncture and collected in plastic capillaries with EDTA (*BD Vacutainer K2E EDTA 10.8 mg*, *BD-Plymouth*, UK) for the comet assay.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMNC) were isolated using Histopaque-1077 (*Sigma-Aldrich*, Germany) according to the protocol provided by the manufacturer. Blood samples were layered on top of the cold Histopaque-1077 (1:1 v/v ratio). Samples were then centrifuged at 400 x g for 30 minutes at 20 ºC in the Sigma 3 – 16 K (*Sartorius*, Germany) centrifuge. Middle layer (contained cells of interest) was transferred to a new falcon tube that was then filled with PBS (*Sigma-Aldrich*, Germany) to the 12 mL mark. The mixture was centrifuged at 250 x g for 10 minutes at 20 ºC. Supernatant was removed and cell pellets were resuspended in 5 mL of PBS. Centrifugation was repeated under the same conditions; supernatant was removed, and cell pellets were resuspended in 3 mL of RPMI-1640 buffer (*Sigma-Aldrich*, Germany) and 10% FBS (*Sigma-Aldrich*, Germany). Samples were then incubated for 24 hours in the MCO – 18 AIC CO_2 incubator (*Sanyo*, Japan) at 37 ºC and 5% CO_2 atmosphere.

Preparation of the composite material

Four commercially available dental composites (suppliers remain a secret due to legal issues) were tested. About 0.03 g of each composite were taken and compressed between two glass slides to form a 2 mm thick plate. Plates were then irradiated for 20 seconds by the Woodpecker Curing Light LED.B lamp (*Zhengzhou Shengxin Medical Instrument Co.,* China). Polymerized materials were placed into falcon tubes containing 500 µL of previously incubated PBMNCs. Samples were incubated for 24 hours at 37 ºC and 5% CO_2 atmosphere.

The single gel electrophoresis (comet assay)

The DNA single-strand breaks were determined in isolated peripheral blood mononuclear cells by means of single-cell electrophoresis as described. 100 µl of fresh isolated PMNCs were mixed with 200 µL of preheated to 37 ºC 0.75% low-melting agarose (*Sigma-Aldrich*, Germany). The mixture (100 µL) was placed on an agarose-precoated (1%, type III, *Sigma-*

Aldrich, Germany) microscope slide and covered with 24 x 40 mm cover slides (*Biosigma*, Italy). The slides were kept on ice up to the solidification of agarose. The cell membranes were lysed in cold lysing solution (2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris, pH 10 (*AppliChem*, Germany), 1% Triton-X 100, 5% DMSO [Sigma]), for 1h. The slides were then placed in a horizontal tank filled with fresh electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.2) for 40 min in darkness to allow DNA to unwind before the electrophoresis (30 min at 300 mA, 1 V/cm and 4 ◦C). Afterwards, the slides were dried for 1 min, washed twice for 5 min with 0.4 M Tris buffer (pH 7.5) for neutralization and fixed in ice-cold 96 % ethanol for 10 min. Slides were dried and stained with ethidium bromide and analyzed with a fluorescence microscope (Leica DFC295) equipped with 515–560 nm excitation filter and 590 nm barrier filter. Cells were visually graded into 5 classes ($A_0 – A_4$) (according to Collins et al., 1993 and 1994) from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). DNA damage index (D) in arbitrary units was calculated as follows: $D = A_1 + 2 \times A_2 + 3 \times A_3 + 4 \times A_4$.

RESULTS

Alkaline comet assay results

Peripheral blood mononuclear cells were isolated from fresh whole blood samples taken from 10 healthy subjects. Dental composites were shaped into 2 mm thick plates and irradiated by LED light with the exposure time of 20 s (recommended by material suppliers). Four different commercially available dental composite samples were incubated with isolated PMNCs for 24 h. Comet assay was performed to evaluate levels of DNA damage. An increase in DNA damage levels was observed after the incubation of each sample containing a dental composite. Control samples were treated under the same conditions as other samples, and no significant differences were observed. There were slight deviations between the results of each material, but overall results followed the same trend. Lowest levels of DNA damage were observed in samples incubated with Composite 2. Composites 3 and 4 showed similar and the highest levels of DNA damage (Figure 5).

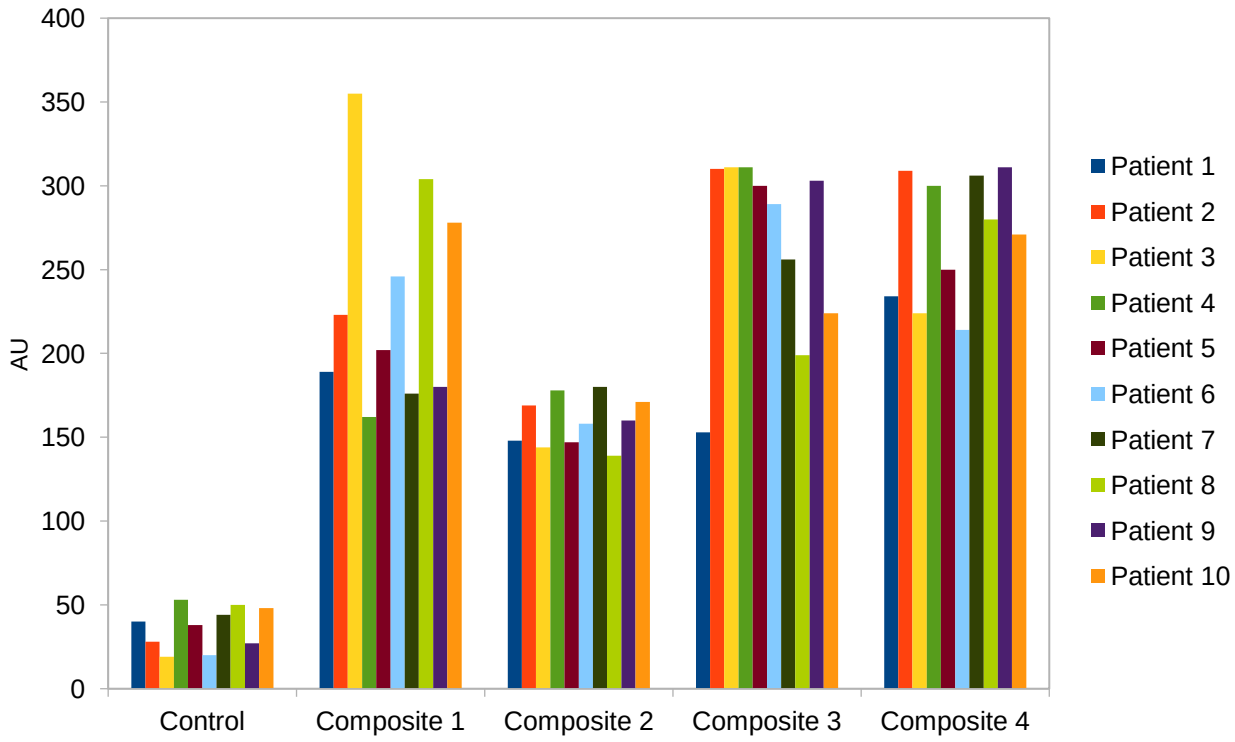

Figure 5. Summary of comet assay results visualizing the data of 10 blood samples from different people treated by 4 different dental composites.

Statistical analysis of comet assay results

Statistical analysis was performed using the commercially available software GraphPad Prism. Mean values of DNA damage levels and standard deviations (SD) were calculated for each sample group (Figure 6). One-way ANOVA test was performed and statistical significance ($p < 0.05$) was reached.

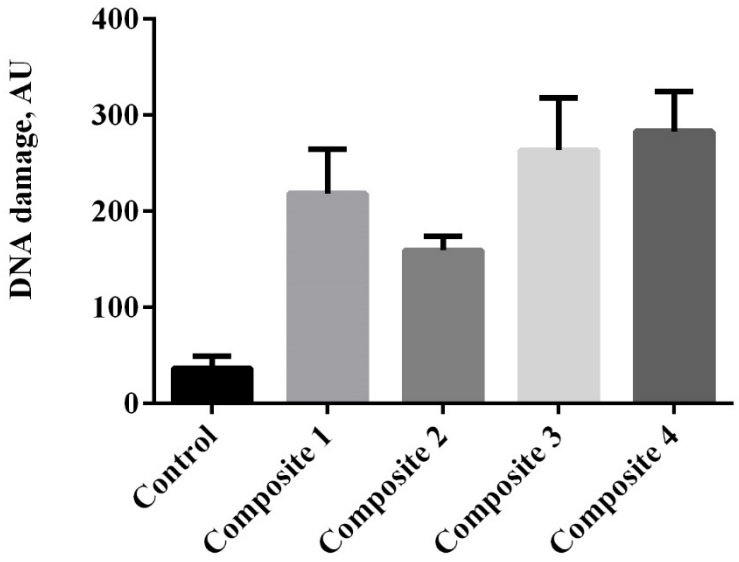

Figure 6. Level of DNA damage (arbitrary units) in PBMNCs. Data are presented as mean ± SD. $F = 67.80$; $p < 0.0001$

Interquartile ranges (IQR) were also calculated and data was summarized in Table 1. Additional two-tailed t tests comparing each study group with the control group were performed. Data from each test (Table 2) showed that statistical significance was achieved indeed.

Table 1. Descriptive statistics data

	Control	Composite 1	Composite 2	Composite 3	Composite 4
Number of values	10	10	10	10	10
Minimum	19.00	162.0	139.0	153.0	214.0
25% Percentile	25.25	179.0	146.3	217.8	246.0
Median	39.00	212.5	159.0	290.0	290.0
75% Percentile	48.50	254.0	172.8	304.8	309.5
Maximum	53.00	304.0	180.0	311.0	355.0
Mean	36.70	218.4	159.4	263.6	283.0
Std. Deviation	12.46	46.28	14.67	54.47	41.99

Table 2. Data of the two-tailed t tests comparing each study group with the control group

Column B	Composite 1	Composite 2	Composite 3	Composite 4
vs.	vs,	vs,	vs,	vs,
Column A	Control	Control	Control	Control
Unpaired t test				
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed
t, df	t=11.99 df=18	t=20.16 df=18	t=12.84 df=18	t=17.78 df=18
Difference between means	181.7 ± 15.16	122.7 ± 6.087	226.9 ± 17.67	246.3 ± 13.85
95% confidence interval	149.9 to 213.5	109.9 to 135.5	189.8 to 264.0	217.2 to 275.4

DISCUSSION

Above a certain level, BPA was considered as a potential inducer of adverse effects. It is now clear that noxious effects are detectable even below a very low dose. The lower dose inducing cell damage is determined by the no observed adverse effect level (NOAEL). It was evaluated by the Food and Drug Administration to be as low as 5 mg BPA/kg body weight (bw)/day. However, according to safety authorities and protection agencies, the tolerable daily intake (TDI) considered as a reference would be a dose of 0.05 mg/kg bw/day. The issue of the dose is still a matter of debate, but it is clear that doses below the NOAEL have significant effects. According to Moon et al. (Moon et al., 2012) doses of BPA below the NOAEL induce mitochondrial dysfunctions in the liver and are associated with an increase in oxidative stress and inflammation. BPA-treated patients may not present immediate adverse effects. Two generations later, epigenetic effects might appear and noxious effects might influence some induced pathologies appearing at the third generation or later, even in BPA-untreated patients (Gayrard et al., 2013).

It was recently shown that the possible high systemic bioavailability of BPA (70–90 %) is controlled by sublingual supervision. Along this line, the transmucosal absorption of BPA within the oral cavity led to much higher BPA internal exposure than the absorption resulting from the gastrointestinal tract. This focuses on the responsibility of BPA released from dental restorative material. The absorption through the oral mucosa may be an efficient systemic entry route, more efficient than orogastric gavage (Gayrard et al., 2013).

Since 40 years the pulp response to bisphenol A-releasing restorative materials was investigated. Comparison was made between a methyl methacrylate monomer and a dimethacrylate thinner material (Auvenshine and Eames, 1972). Direct pulp capping increases the blood vessel density near the pulp exposure (Mantellini et al., 2006).

BPA is at the origin of undesirable effects. It was concluded that the resin component BPA has the capacity to inhibit macrophage function and modulate immune and inflammatory response in dental pulp and periapical tissues (Segura et al., 1999)

Olea et al. (Olea et al., 1996) concluded that normal values and concentrations found after BPA treatment were reported in body fluids. In blood no BPA was found in blood samples prior or after dental treatment. In saliva collected 1 h before the application of cured sealants. After treatment, all saliva samples contained BPA in amount ranging from 90 to 931 μg. In control patients, BPA was detected in the saliva of all patients prior to the placement of the sealants and ranged between 0.07 and 6.00 ng/ml at baseline. Three hours after treatment, the salivary concentration peaked and returned to the baseline level within 24 h. Low peak levels were 3.98 ng/ml (one sealant application alone), whereas 9.08 ng/ml in the high-dose group

(more than four sealants) (Zimmerman-Downs et al., 2010). Altogether, the different clinical studies available conclude that the highest level of BPA reported in saliva from dental sealants is more than 50,000 lower than the lethal dose 50 (LD50) values reported for BPA. This allows some researchers to conclude that human exposure to BPA from dental resins is minimal and poses no known health risk (Rathee et al., 2012).

This contradicts some findings establishing that some low-dose effects of BPA are released from dental resins through salivary enzymatic hydrolysis of BPA derivatives. BPA is detectable in saliva for up to 3 h after resin placement. Exposure to low doses of BPA resulted in significant alterations in gland morphology, which varied to subtle effects on mammary gland development when the exposure period occurs in adulthood, leading from precancerous to cancerous lesions. Prenatal exposure to relevant doses of BPA increases the number of intraductal hyperplasia and ductal carcinoma (Acevedo et al., 2013).

Short-term treatment with BPA leads to metabolic abnormalities in insulin-sensitive peripheral tissues. Mice treated with BPA were insulin resistant and had increased glucose-stimulated insulin release. It was concluded that short-term treatment with low dose of BPA slows down whole body energy and disrupts insulin signalling in peripheral tissues. Therefore, BPA can be considered as a risk factor for the development of type 2 diabetes (Batista et al., 2012).

Exposure of 3T3-L1 preadipocytes for 14 days to BPA reduced the amount of triglyceride accumulation and suppressed the gene transcription of the lipogenic enzyme lipoprotein lipase. BPA can reduce triglyceride accumulation during adipogenesis (Linehan et al., 2012).

Our results show that given dental composites have a certain degree of genotoxicity. At the current state material genotoxicity cannot be explained by the presence of any chemical component. However, one can assume that chemical components have a certain effect on the level of the DNA single strand breaks in isolated human peripheral blood mononuclear cells.

Other studies of dental composite genotoxicity show controversial results. Keinasser et al., 2006 states that a significant DNA damage is observed when human salivary glands and lymphocytes are targeted by TEGDMA, UDMA and HEMA while detecting potential treats of carcinogenesis. In the same study the mentioned compounds are considered to be potent agent of the increased tumour risks. Another evidence of BPA derivative genotoxicity is mentioned in Drozdz et al., 2011, stating that bis-GMA has an ability to induce DNA double-strand breaks in human lymphocytes, interfering with the cell cycle and increasing the rate of cell apoptosis.

A correlation between levels of DNA damage and potentially toxic monomers have been found. Bis-GMA assumed to have the lowest affinity to DNA, causing less damage, followed

by UDMA and TEGDMA, and with the most toxic monomer being HEMA. According to Urcan et al., 2010 such increase in potency to cause double stranded DNA breaks was studied on human oral cavity cells, nevertheless, the observed genotoxicity was still below the critical value. On the other hand, a study by Tauböck et al., 2017 shows that none of the bulk-fill composites had no effect of the induction of DNA damage or causing any other visible chromatin alterations.

Current significant data regarding this topic are scarce. In order to find any correlations between monomers in each material and levels of DNA damage further investigation of additional variables, such as, different exposure time, the amount of material and polishing is required. Possibly, more advanced techniques should be applied to fill the knowledge gap of this topic.

CONCLUSIONS

To summarize this project, one can first say that the chosen dental composites have a genotoxic effect when in contact with human peripheral blood mononuclear cells. Comparing results from the control group with study groups, levels of DNA damage have at least 4-fold increase for each investigated material. Also, all experiments are considered to have great reproducibility and precision.

Current results do not provide with an insight of the effect of each possibly toxic BPA derivative or BPA itself. To correlate the results for each chemical compound additional studies are required.

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