Biocompatibility of Dental Adhesives

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Chapter 2

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Additional information is available at the end of the chapter

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Abstract

The accomplishment of developing a truly adhesive bond between a restorative material and the natural tooth structures is the goal of adhesive dentistry. Dentine adhesive systems come into close contact with dental and oral tissue, especially the pulp and gingival cells. Due to this close and long-term contact, adhesives should exhibit a high degree of biocompatibility. Biocompatibility is one of the most important properties of dental materials, and adhesives are no exception. It has been long demonstrated that different components of adhesives can be released. Numerous *in vitro* investigations have shown that released monomers and other components can cause damage to cultured cells. In addition, many *in vivo* studies have shown that uncured components which reach the pulpal space cause inflammatory response and tissue disorganization. Only a combination of various *in vitro* and *in vivo* tests can provide an overview of the interaction of biomaterials with the host. Therefore, it is necessary on a regular basis to carry out and re-verify the biological compatibility of the increasing number of new dental materials. Adhesives should be biofunctional, protective, and preventive, with health-promoting effects that contribute to a better prognosis for restorative treatments and its biocompatibility.

Keywords: biocompatibility, cytotoxicity, dental adhesives, genotoxicity, *in vitro* assays, *in vivo* assays

1. Introduction

The use of composite dental filling materials along with adhesive techniques has revolutionized today's dental practice. Dental adhesive systems are used to improve contact between restorative material and the walls of the prepared cavity of tooth, in order to increase retention as well as to minimize bacterial leakage. Their purpose is based on a twofold adhesion, adhesion to composite fillings, and bonding to enamel and dentine [1–3].

The introduction of the acid-etch technique by Buonocore in 1955 was the genesis of adhesive dentistry. Adhesive systems are constantly being developed with elaborate and complex chemistry. As a result of technological progress, the large number of new materials on the market has been appeared. Adhesive systems are solutions of resin monomers that make the resin-dental substrate interaction achievable. They are composed of monomers with hydrophilic and hydrophobic groups, organic solvents, initiators, inhibitors or stabilizers, and inorganic fillers. The proportional composition and the chemistry of these ingredients have been different between the different classes of adhesives [1, 4]. Dentine adhesives are currently available as three-step, two-step, and single-step systems, depending on how the three basic steps of etching, priming, and bonding to tooth substrate are accomplished. Modern adhesive strategies depend on how adhesive systems interact with the smear layer dissolving it or making it permeable. Consequently, adhesives can be classified into two types: total-etch (also known as etch-and-rinse) and self-etch adhesive systems [4]. The main difference between them is that the etch-and-rinse adhesives use 37% phosphoric acid for pretreatment of hard dental tissues before application of dental bonding agent, whereas the self-etch adhesives do not require a separate etching step, as they contain acidic monomers that simultaneously condition and prime the dental substrate [2, 5]. Etch and rinse adhesive systems are used either through two or three clinical application steps. First step always involves application of the conditioner or acid etchant on both enamel and dentine. According to the number of clinical application steps, self-etch adhesives are divided into two-step and one-step systems [6].

In order to achieve clinical success, it is necessary for dental adhesives to provide good physical properties. Clinical success of adhesive procedures depends not only on the dental adhesives but also on variables such as substrate condition and composition, clinical technique, restorative material, cavity shape and size, and polymerization quality [6]. However, due to the fact that dental adhesives are in close and extended contact with vital dentine, biocompatibility becomes a property of supreme [5, 7, 8]. The biocompatibility of the adhesive estimated through screening with simple *in vitro* assays, mostly based on cell cultures, is followed by more comprehensive studies on experimental animals and usage tests and ends with clinical studies [9]. The scientific evidence on adhesives is contradictory. Some authors claimed that they are very safe and can be used even in direct contact with the pulp, whereas others believe that they are not suitable for direct pulp capping due to reported associated symptoms of persistent inflammation [10, 11]. Another researchers claim that dental adhesive systems contain certain components that can be released into the oral environment and show genotoxic, allergenic, cytotoxic, estrogenic, and mutagenic activity and that they alter lipid metabolism, glutathione (GSH) concentration, reactive oxygen species (ROS) production, cell cycle, energy metabolism, and mitochondrial activity [2, 5, 7, 12–16]. There are a number of *in vitro* studies that have proven cytotoxic and genotoxic activity of dentine-bonding agents on the cells of human origin (peripheral blood lymphocytes, lung fibroblasts, pulp cells, gingival fibroblasts and oral epithelial cells, pulp cells, dentine, lymphocytes, and leucocytes) [5, 17–20]. Most *in vivo* research has been performed on monkeys and rodents, whereas the rare studies conducted on humans [19].

1.1. Biocompatibility of dental materials

Biomaterial is a substance that is used for a long period within the body with the aim of treating or replacing of tissue, organs, or their functions. Each dental material must be biocompatible or able to function *in vivo* without eliciting an intolerable response in the body either locally or systemically. There are only few materials for which could be said that are biologically inert since most of them contain a potentially harmful or irritating ingredients [9, 21].

Biocompatibility is a measure of body's biological response to a material used in specific application. It is a property of a material to interact with its environment. The biological response to a material could be modified if any change occurs in the host or in the application of the material. Therefore, evaluation of any new material intended for human use requires data from systematic testing to ensure that the benefits provided by the final product will exceed any potential risks produced by device materials [9]. Biocompatibility includes the physical, mechanical, and chemical properties of materials, as well as potential cytotoxic, genotoxic, mutagenic, and allergenic effects. According to EN 1441 (European standard for Risk Analysis, 1996), biocompatible material must not be harmful to the host organism [22]. A characteristic of the material to interact with the biological tissue and thus create a stable connection is essential for biocompatibility. The biocompatibility of material is manifested through a number of parameters: (a) cytotoxicity (systemic and topical), (b) genotoxicity, (c) mutagenicity, (d) carcinogenicity, and (e) the immunogenicity [23].

The toxicity of material is described as the ability of a chemical substance to cause harmful health effects and damage the biological system. It might be local or systemic. Local toxicity occurs at the place of application, as opposed to systemic toxicity where the adverse response occurs distant from its entry point [23, 24]. The term of cytotoxicity is used to describe the cascade of molecular events that cause functional and structural damage to cells. Throughout the years, various assays and protocols were developed to test the cytotoxic effects of biomaterials. The rationale behind doing a cytotoxicity test is to determine how a material sample affects a particular cell type. The primary criteria of these tests are that material must not affect the cell number, cell growth, genetic integrity, membrane integrity, genetic expression, and enzymatic activity of the cells. The cytotoxicity assays are broadly categorized into viability assays, survival assays, metabolic assays, transformation assays, and inflammation assays [25]. The cytotoxicity is related to the damage of the individual cells, for example in cell cultures, and determines the cell viability. There are two modes of cell death, apoptosis (programmed cell death) and necrosis (accidental cell death). They are different significantly in the mechanisms, outcomes, and also in morphology, biochemistry, and biological features. Necrosis occurs as a result of significant damage of the cell, caused by physical or chemical agent. The loss of integrity of the membrane is a hallmark of necrosis. The membrane permeability of damage cell increases, and damage cell cannot control balance of the fluids and ions. It is accompanied by cellular swelling and loses of integrity of organelles. The release of cytoplasmic contents could lead to an inflammatory response in surrounding organs and tissue. Apoptosis could be defined as caspase mediated cell death. Apoptotic cells can be identified based on following morphological features: cytoplasmic and nuclear condensation, chromatin cleavage, formation of apoptotic bodies, maintenance of an intact plasma membrane, exposure

of surface molecules targeting cell corpses for phagocytosis and also on the proteolytic activity of certain caspases because these enzymes mediate the process of apoptotic cell death [23, 26, 27]. The primary criteria of these tests are the following: the material must not affect the cell number, cell growth, genetic integrity, membrane integrity, genetic expression, and enzymatic activity of the cells [25].

Genotoxicity is described as adverse effects on the genomic material and may be caused by DNA damage without direct evidence for mutations [23]. Genotoxic effect of a material can induce changes in the genome that disrupt its integrity or function. Depending on the intensity of that effect, the cell could recover, start neoplastic growth, or die [28]. The long-term exposure to low concentrations of the substance may develop neoplasia and death of the whole organism by the effects on the genetic material. Transfer of genetic damage to the next generation of cells can be avoided by programmed cell death (apoptosis) [9, 28].

Transition of genetic injury to the next generation is called mutagenicity. Mutagenicity and carcinogenicity are not the same entities. Carcinogenicity arises from several mutations, which means that all mutagenic events do not lead to tumour development. Mutagenicity serves as an indicator of 'possible' carcinogenicity of substances that damaged DNA. A variety of different methods, mainly *in vitro* assays (Ames test, micronucleus, and hypoxanthine phosphorybosyl transferase (HPRT) test on mouse lymphoma cells), are used in the assessment of the mutagenic potential of materials [9, 23].

The immunogenicity is the ability of a substance to induce an immune response. An allergic reaction to certain substances can be initiated if the organism was sensitized previously. The dosage that could cause the allergic reaction are generally significantly lower than those that could cause toxic reactions. Immunotoxicological screenings are used to identify the influence of substances or materials to the various components of immune system [23].

Biomaterials must meet several criteria before they are put in use, and measuring of the biocompatibility of a material is not simple. It is not possible to biologically characterize a material using a single test. Different characteristics can be explored through both *in vitro* and *in vivo* assays. *In vitro* biocompatibility tests are conducted outside of a living organism with the purpose of simulating the biological response to a material. The influence of the material is determined by measuring the size, growth factors, metabolic functions as well as other functions of a cell treated with a tested material. These tests have lots of advantages; they are standardized, relatively low cost, quick, and easily reproducible. But their clinical relevance is questionable because they provide limited answers due to lack of biological and physiological components [21, 29]. *In vitro* studies are primarily performed to evaluate the cytotoxicity (cell damage) [12, 18, 19, 30] or genotoxicity (specific DNA damage or chromosomal aberrations) [2, 5, 7, 31] of dental materials. *In vivo* biocompatibility testing has provided the influence of tested material in a living organism. Investigations are most commonly conducted on animals. Mostly, tested materials are implanted in the body, followed by monitoring on their influence on a living organism. Thus, it is possible to evaluate the many complex interactions between biological systems and materials, either in the surrounding tissue or at a remote site in an organism [9, 23]. Animal experiments for cytotoxicity tests of dental materials are time-consuming, expensive, and the theme of extensive public discussions [32]. Clinical *in vivo*

assays are more reliable, since the tested material is directly applied in the body of volunteers in their ultimate purpose. The results obtained from these studies are of particular interest. However, clinical studies have numerous limitations, are expensive, long term, and difficult to control since many external variables can influence results. Therefore, it is challenging to interpret the obtained results [9, 23].

2. Materials and methods

2.1. Blood sampling

Evaluation of the potential genotoxicity of dental adhesive systems and combination of adhesive systems and composite materials were performed on human leukocytes. The donors were two males and four females with ages ranging from 25 to 32. They were not smokers and had not been exposed to any physical or chemical agents that might have interfered with the results of the genotoxicity testing in the 12-month period prior to blood sampling. The donors were acquainted with the purpose of the study and signed permission for the blood samples to be used for scientific purposes. A peripheral blood sample was collected under sterile conditions by venipuncture into heparinized tubes (Becton Dickenson, Plymouth, UK). The study was approved by the Ethical Committee of the School of Dental Medicine, University of Zagreb, Croatia [3, 5].

2.2. Preparation of materials and cell culture treatment

Three dental adhesives were tested: AdheSE (Ivoclar Vivadent, Schaan, Liechtenstein), G-bond (GC, Tokyo, Japan), and Adper Single Bond (3M ESPE, St. Paul, MN, USA), in their polymerized and unpolymerized form. Also were tested aforementioned dental adhesive in polymerized form in combination with composite material from same manufacturers: Tetric EvoCeram (Ivoclar Vivadent, Schaan, Liechtenstein), Gradia Direct Anterior (GC, Tokyo, Japan), and Filtek Z250 (3M ESPE, St. Paul, MN, USA). Six different combinations of two shades of composite resins and adhesives were examined: Gradia Direct Anterior A1 + G-Bond, Gradia Direct Anterior A3.5 + G-Bond, Filtek Z250 A1 + Adper Single Bond, Filtek Z250 A3.5 + Adper Single Bond, Tetric EvoCeram A1 + AdheSE, and Tetric EvoCeram A3.5 + AdheSE.

In order to test the genotoxicity of non-polymerized materials, the dental adhesives were placed in previously weighed bottles (Sartorius BLG10S, Goettingen, Germany). The mass of each dental adhesive was calculated from the difference in weight of empty and full bottles. For each 0.1 g of dental adhesive, 1 ml of saline solution was added (0.9% NaCl, Sigma, St. Louis, MO, USA) for the purpose of elution.

In order to test the genotoxicity of polymerized dental adhesives, each one was polymerized under aseptic condition in accordance with the manufacturer's instructions using an Elipar TriLight halogen curing unit (3M ESPE) from a 2 mm distance for 40 s. To ensure complete polymerization, only two drops of an adhesive were cured at a time. After polymerization, the dental adhesives were weighted, fragmented, and transferred into bottles. We tended to use

the same masses of dental adhesive samples (1 g) regardless of whether they were polymerized or non-polymerized. Each dental adhesive eluate was tested after 1 h, 1 day, and 5 days in two different dilutions of eluate (1: 102 and 1: 104) for each time point.

To assess the genotoxicity of polymerized dental adhesives and their compatible dental composites from same manufacturers, the following was conducted: 20 ± 0.2 μl of each adhesive system was placed on the Mylar strip (Contour™ Strips, Ivoclar Vivadent) and photopolymerized by a halogen curing unit Elipar TriLight in standard mode (800 mW/cm^2) for 20 s. The adhesives were handled exactly by the manufacturers' instructions. Subsequently, 0.025 ± 0.003 g and 0.05 ± 0.002 g of each composite resin were placed on top of the bonding material, covered with another Mylar strip, and mechanically pressed to obtain a 2 mm thick layer. Resin composite samples (n = 2) were for 40 s. The light curing tip was flush pressed onto the Mylar sheet on top of the composite samples. Thereafter, the polymerized composites were separated from the Mylar sheets, and the samples of each material combination were placed in a plastic sterile tube (Greiner Labortechnik Co., Ltd., Frickenhausen, Germany) with saline solution (0.9% NaCl, Sigma, St. Louis, MO, USA) to be eluted for 1 h, 1 day, 7 days, and 30 days. For each 0.1 g of dental composite materials, 1 ml of saline solution was added for the purpose of elution.

Cultures for cytogenetic testing were set up at the end of the elution period. One millilitre of primary leukocyte culture containing 5.6 × 106 cells was introduced into 9 ml of F-10 HAM's medium (Sigma) without the addition of fetal bovine serum or mitogen. Leukocytes were treated with 1 and 100 μl of eluates obtained from each of the tested dental adhesives to, respectively, simulate final mass concentrations of 0.1 and 10 mg of materials/ml. While composite-adhesive elusion solutions were discarded, and the samples were carefully transferred to a new sterile tube (Greiner Labortechnik Co., Ltd., Frickenhausen, Germany), with added 1 ml of primary leukocyte culture (density of 100 cells/μl) and 5 ml of RPMI 1640 (Gibco-Invitrogen, Carlsbad, CA, USA). After that, leukocytes were treated with 0.025 g and 0.05 g of each of the composite/adhesive combinations to, respectively, simulate final mass concentrations of 4.16 and 8.33 mg of sample/ml. Control leukocyte cultures were exposed for 48 h to a clean medium RPMI 1640 (combination of adhesives and composites) and a saline solution (0.9% NaCl, Sigma). Each DNA damage experiment included also positive control, which was hydrogen peroxide, 60 μl, for 15 min on ice. After the treatment period (48 h at 37°C in a 5% CO_2 atmosphere), cultures were centrifuged for 10 min at 70 g, the supernatant was discarded, and cells were transferred into a sterile tube (Nange Nunc International, Naperville, IL, USA). They were resuspended and sampled for vital staining and the comet assay. Each DNA damage experiment included also positive control, which was hydrogen peroxide, 60 μl, for 15 min on ice [3, 5].

2.3. Cytotoxicity testing

Leukocyte viability was tested using the trypan blue exclusion technique [33]. Cell suspension was mixed with 0.4% trypan blue (Sigma) and analysed using an Olympus light microscope (Olympus, Tokyo, Japan) under 100× magnification. For each test group, 1000 leukocytes were

analysed by counting unstained (viable) cells. Blue-coloured cells were considered to be nonviable [3, 5].

2.4. Comet assay

The comet assay was carried out under alkaline conditions as described by Singh et al. [34]. All chemicals used to perform the comet assay were obtained from Sigma. Sediment containing leukocytes was suspended in 100 μl of 0.5% low-melting-point agarose. This agarose layer was sandwiched between a layer of 0.6% normal melting point agarose and a top layer of 0.5% low melting point agarose on fully frosted slides. Slides were coded and kept on ice during polymerization of each gel layer. After solidification of the 0.5% agarose layer, slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% DMSO) at 4°C. After 1 h, the slides were removed from the lysing solution, drained, and placed in an electrophoresis buffer (0.3 M NaOH and 1 mM Na2EDTA, at pH 13) at 4°C for 20 min to allow the DNA to unwind. Electrophoresis was conducted on a horizontal electrophoresis platform in fresh, chilled electrophoresis buffer for 20 min at 300 mA and 19 V. After electrophoresis, slides were neutralized with Tris-HCl buffer (pH 7.5) three times for 5 min each and stained with ethidium bromide (20 μg/ml) for 10 min. Two slides per material per concentration per polymerization form per time point were analysed using an Ortoplan epifluorescence microscope (Leitz, Wetzlar, Germany) at 250× magnification (**Figure 1**). One hundred comets per slide were analysed by the comet assay II automatic digital analysis system (Perceptive Instruments, Halstead, UK) by measuring the tail length and intensity (% DNA). For the purpose of the analysis, the following were ignored: the edges and eventually damaged parts of the gel as well as debris, superimposed comets, comets of uniform intensity, and comets without a distinct head (i.e., 'clouds', 'hedgehogs', and 'ghost cells') [3, 5].

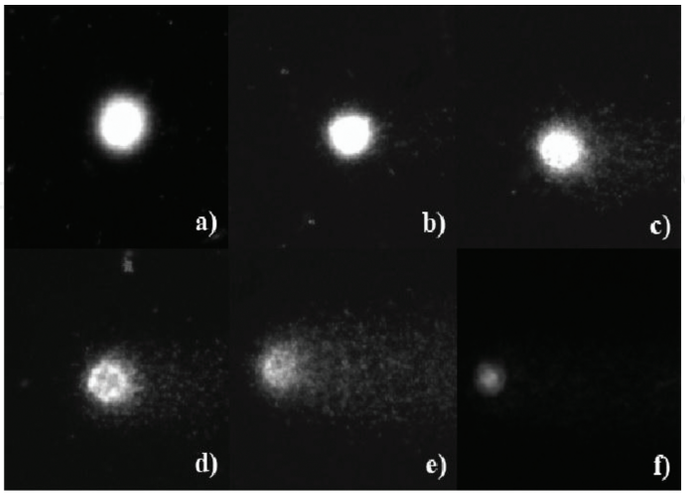

Figure 1. Representative microscopic appearances of comet, taken under an Ortoplan epifluorescence microscope: (a) undamaged cell; (b–e) damaged cells; and (f) apoptotic cell.

2.5. Statistical analysis

The comet test results were tested by the Kruskal-Wallis test to determine the statistical significance. The level of significance was set to 0.05. All calculations were performed using the commercial software, Statistica 7.0 (StatSoft, Tulsa, OK, USA).

3. Results

Before testing, concentrations of the dental adhesives were selected based on the cytotoxicity results. The trypan blue exclusion test showed that the viability was >85% for each material at a dilution of >1:102 and greater than 90% for each material combination at a concentration of 8.33 mg/ml. According to these results, other dilutions and concentrations are considered to be used without any risk. The pH value in the cell culture was always between 7.19 and 7.4, which is, according to the manufacturer, the regular pH value.

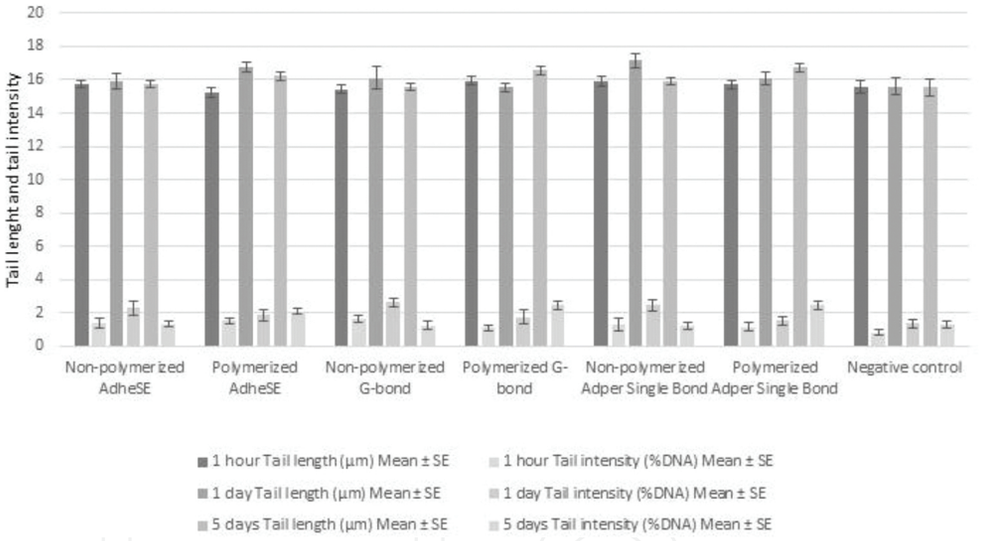

Figure 2. Tail length and tail intensity (mean values and standard deviations) of leucocytes exposed to a 10^{-2} elution of dental adhesives.

The comet test determined the level of primary damage to DNA molecules of leukocytes after treating them with different dilutions of eluates of dental adhesives, depending on the length of time they were rinsed in the saline solution. In the present study for each dilution, elution duration, and polymerization state, 100 comets were analysed. **Figures 2** and **3** showed two basic parameters of the comet tail, length, and the intensity of its fluorescence, of leukocytes exposed to a 10^{-2} and 10^{-4} elution of polymerized and unpolymerized dental adhesives. None of the tested dental adhesives revealed a statistically significant increase in tail length or tail intensity in treated leukocytes, independent of the applied dilution, elution duration, and polymerization form. **Figures 4** and **5** represent the results of comet assay parameters (mean values and standard deviations) in human leukocytes during exposure to tested combinations

of adhesive and composite resins in two different shades (A 1 and A 3.5), in concentrations 4.16 mg/ml and 8.33 mg/ml. Obtained results showed statistically higher increase in tail length after 1 day for Tetric EvoCeram in shade A1 for lower concentration (4.16 mg/ml) (15.3 ± 2.52, negative control 14.7 ± 2.85), and after 1 h (15.3 ± 4.70, negative control 14.7 ± 2.85) and 1 day (15.2 ± 9.10, negative control 14.7 ± 2.85) or same material for higher concentration (8.33 mg/ml). Concerning the tail intensity only Gradia Direct in the shade A 3.5 + G-Bond showed statistically higher percentage of DNA in tail in concentration 8.33 mg/ml eluted for 1 day (3.2 ± 5.64, negative control 1.2 ± 3.71) [3, 5].

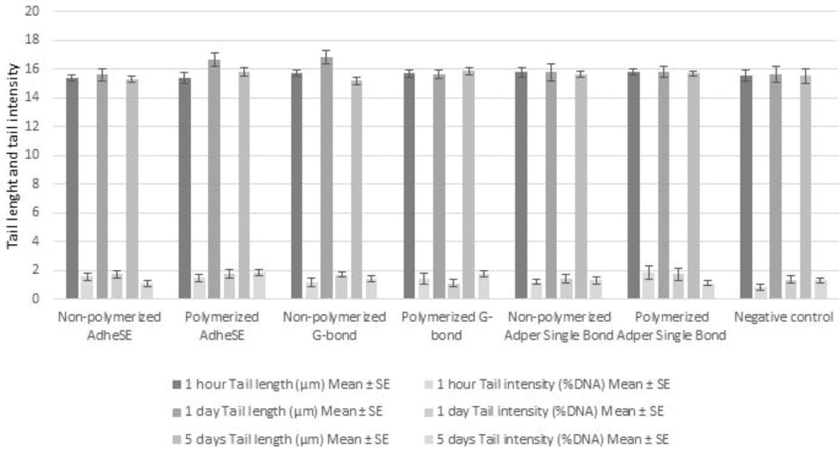

Figure 3. Tail length and tail intensity (mean values and standard deviations) of leukocytes exposed to a 10^{-4} elution of dental adhesives.

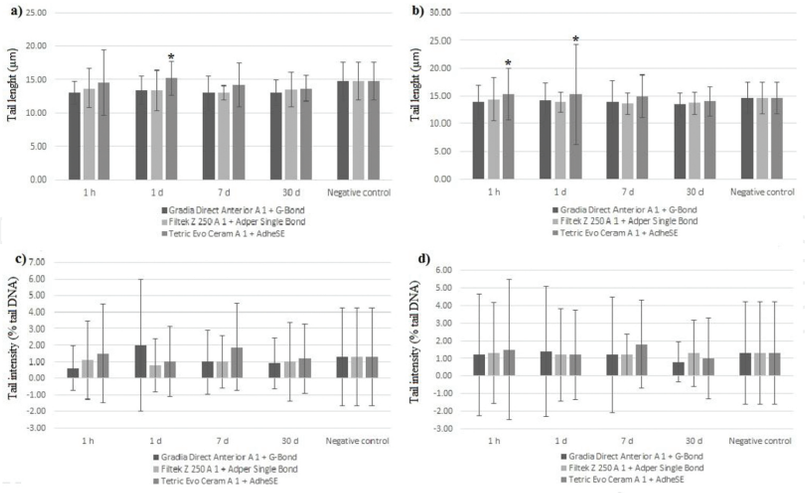

Figure 4. Comet assay parameters (mean values and standard deviations) in human leukocytes during exposure to tested combinations of composite resins A 1 and adhesive: (a) tail length for material combinations in a concentration of 4.16 mg/ml; (b) tail length for material combinations systems in a concentration of 8.33 mg/ml; (c) tail intensity (% tail DNA) for material combinations in a concentration of 4.16 mg/ml; and (d) tail intensity (% tail DNA) for material combinations in a concentration of 8.33 mg/ml. Asterisk denotes material combinations significantly different from the negative control ($p < 0.05$).

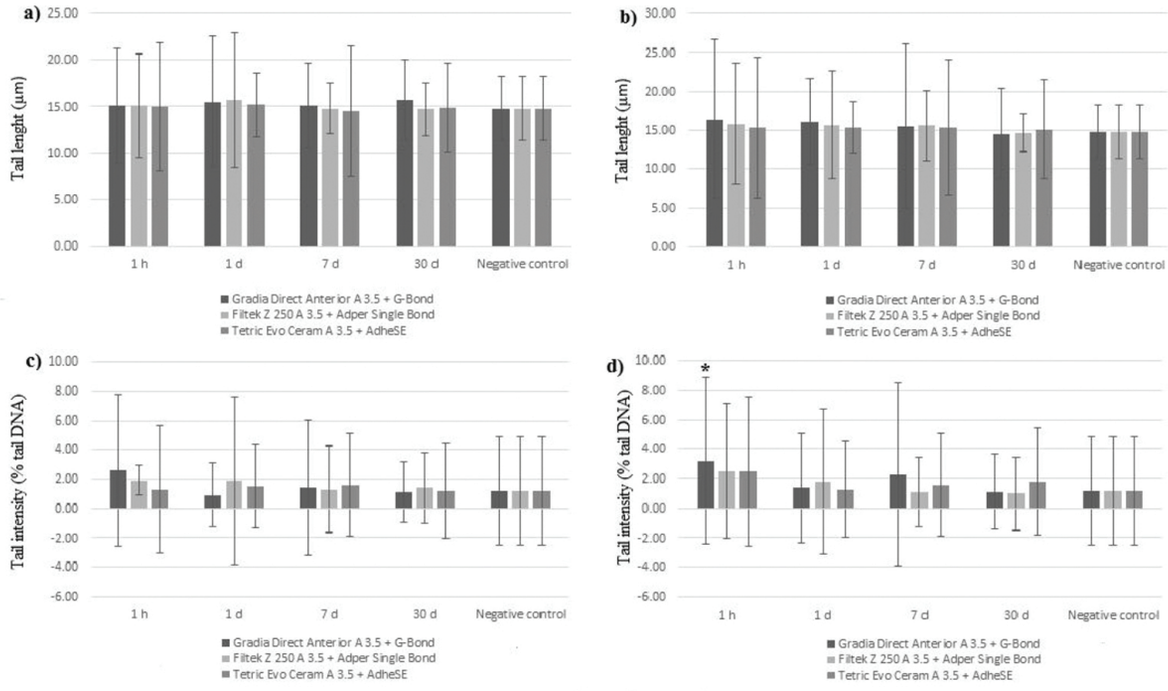

Figure 5. Comet assay parameters (mean values and standard deviations) in human leukocytes during exposure to tested combinations of composite resins A 3.5 and adhesive: (a) Tail length for material combinations in a concentration of 4.16 mg/ml; (b) tail length for material combinations systems in a concentration of 8.33 mg/ml; (c) tail intensity (% tail DNA) for material combinations in a concentration of 4.16 mg/ml; (d) tail intensity (% tail DNA) for material combinations in a concentration of 8.33 mg/ml. Asterisk denotes material combinations significantly different from the negative control ($p < 0.05$).

4. *In vitro* biocompatibility testings of dental adhesives

Contemporary dental adhesive systems are used to improve the contact between composite restorative materials and the walls of the prepared tooth cavity. As these materials come in close and prolonged contact with vital dentine, their influence on pulp cells is critical [31]. Adhesive systems that create a stable relationship with biological tissues and allow both healing and tissue differentiation are considered biocompatible.

The effective toxicity of adhesives and its components is reduced but often not eliminated by the presence of dentine. Adhesive systems are placed on etched dentine (total-etch adhesives) or on cut dentine (self-etch adhesives) that is permeable. Since the available adhesive systems are not able to hermetically seal the deep dentine, in several *in vitro* and *in vivo* studies, it has been demonstrated that after application of bonding agents on the conditioned dentine, residual monomers as well as other components of the adhesive systems may penetrate through the dentinal tubules and reach the dental pulp [18, 35]. The diffusion of chemical products from dentine to the pulp is dependent upon several important variables, including (a) the thickness of the remaining dentine, (b) the surface area of the exposed, (c) the presence or absence of a smear layer, (d) the potency of the microbial products, (e) the rate of pulpal

blood flow, and (f) dentine permeability [36, 37]. Smear layers on dentine have been shown to reduce diffusion through dentine by 25–30%, but most current bonding adhesives use primers that remove the smear layer [38]. Dentine permeability increases with the depth of the cavity, reducing the thickness of the remaining dentine and removing the smear layer, whereas decreases with the age due to physiologic sclerosis of dentine. High permeability increases the toxicity of adhesives by allowing increased diffusion of the released components through dentine [39]. Thicker dentine lowers the concentrations of substance that reach the pulp, keeping these concentrations below thresholds that irritate pulp tissue. It has been demonstrated that 0.5 mm of dentine can reduce material toxicity to 75% and that 1 mm of dentine can reduce toxicity by up to 90% of the control value [40]. Also, the presence of dentine, as biological barrier, can also modify toxicity action due to the inherent characteristics of that tissue such as buffer capacity and hydraulic conductance [41].

The toxicity has been attributed to the release of residual monomers from polymerized adhesive systems due to degradation processes or the incomplete polymerization as well. The degree of conversion of dental bonding systems relies mainly on the wavelength and power intensity of the light curing unit [42]. Polymerization can be inhibited by the presence of oxygen, the presence of intrinsic water from dentine, and the presence of residual solvents in the adhesive [1]. The degree of conversion is an important parameter because it specifies the amount of monomers converted in polymer. Even under ideal conditions, the conversion of monomers to polymers is commonly incomplete, and it is about 55–60% [9]. Unconverted free monomers from polymerized adhesive monomers diluted into saliva after curing as result of degradation may be released into the oral environment where they could produce harmful local effects, as chronic inflammatory reactions of human pulp. Also, it has been demonstrated that they are responsible for many cytotoxic and possible endocrine-disruptive impact. The amount of the monomer release ranges from micrograms to milligrams [24].

Conventional adhesive systems require acid etching of the surface of enamel and dentine as a separate step before their applications. Application of acids to dentine surface removes the smear layer, opens the dentinal tubules, and decalcifies the intertubular and peritubular dentine [43]. Despite paste apprehension about potential toxicity of acid penetration in dentine tubules and pulp space, the interaction of etchants with dentine is limited to the superficial 1.9–5.8 μm. It is unlikely that the acid is directly responsible for pulp injury. The effect of acid on the dentine is limited by the action of hydroxyapatite and collagen which acts as a baffler [41]. Total etching is considered safe if the length of the remaining dentine is more or equal to 0.5 mm. Therefore, total-etching adhesives are recommended in shallow cavities, located in superficial or sclerotic dentine where the permeability of the dentine is low and the thickness of the remaining dentine is adequate to prevent any adverse effects from diffusing materials [36, 44]. After acid etching, a significant increase in dentine permeability due to smear layer removal and opening of dentinal tubules can facilitate the permeation of resin monomers towards pulp, mainly in deep dentine [45]. About et al. [46] concluded in their study that dentine-bonding agents do not affect the cytodifferentiation of secondary odontoblasts when the remaining dentine thickness is 0.7 mm and when the materials are properly polymerized. When adhesives are applied in deep cavities, residual monomers might reach the pulp by

diffusion, and in etched dentine, this penetration tends to be higher. Odontoblasts are typical pulp cells and are the first cells to be damaged by potentially cytotoxic compounds released from dental materials that diffuse through enamel and dentinal tubules. Injury to the odontoblasts results in loss of the capacity of these cells to secrete reactionary dentine, and secretion of dentine bridge by a new generation of odontoblast-like cells [47]. An evaluation of the cytotoxic effect of total-etch two-step adhesive systems showed that both the acidic and non-acidic components of unpolymerized adhesive resins were responsible for high cytotoxic effects on odontoblast-like cells [48].

The most widely used self-etching adhesive systems involve two application steps: the conditioning of dentine and enamel with a self-etching primer, followed by the application of an adhesive resin. There are also one component self-etching adhesives, which contain etch, prime, and bond functions in a single solution. For these adhesive systems, the adhesive resin infiltrates to the same depth as the acidic primer exposed the collagen in dentine [6]. Therefore, the use of self-etching adhesives systems is indicated for young, deep, permeable dentine [36, 49]. da Silva et al. [8] compared the effectiveness and biological compatibility of different generations of dentine adhesives. Their results showed that the one-step self-etch system had the best bond strength performance and was the least toxic to pulp cells, referring to it as a good alternative for specific cases. These results are in accordance with those given by Hashieh et al. [50] who have shown that one-step dentine-bonding agents were less toxic in cell culture than multistep counterparts. Self-etch adhesives have proved to have less cytotoxicity and better tissue response in histological evaluations than those related to conventional adhesive systems, but at this time they are still harmful to the pulp [51, 52].

Any restorative materials that are placed adjacent to vital pulp can induce biological effects. These effects are controlled by the components that are released from the material and the pulpal response to those components. The composition of the adhesive system plays an important role in the toxic effect it produces, and the choice of adhesive system for clinical use should take into consideration its biocompatibility [17]. The usual composition of bonding agents includes resin monomers, initiators, inhibitors or stabilizers, solvents, and sometimes inorganic fillers [2]. It has been reported that dental adhesives release substances that have biological effects (cytotoxicity, carcinogenicity, mutagenicity, and genotoxicity) and toxic potencies [2, 7]. Acidic and nonacidic components of polymerized and unpolymerized adhesives are considered responsible for the cytotoxic effects on the dentine-pulp system [16, 53, 54]. The resins in the adhesive systems are of interest to many researchers, and its biocompatibility has come under extensive scrutiny [12, 31, 55, 56].

The monomers are found to be cytotoxic in cell cultures and to affect the metabolism of the cells. Bisphenol A-glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), hydroxyethyl methacrylate (HEMA), and triethylene glycol dimethacrylate (TEGMA) are considered to be among the most toxic resins [50, 57, 58]. Several *in vitro* studies have indicated that monomers, such as Bis-GMA and UDMA, are strongly cytotoxic to fibroblasts, whereas HEMA and TEGDMA are moderately cytotoxic [55, 59]. Ratanasathien et al. [60] assessed the cytotoxicity of the dentine-bonding components in cell cultures and found that the ranking by toxicity was: Bis-GMA > UDMA > TEGDMA > HEMA after 24 and 72 h of exposure. It is

important to emphasize that synergistic interaction between the multiple components may occur resulting in more cytotoxicity than the individual components would have caused by themselves [50, 60]. A bonding agent that contained Bis-GMA was less cytotoxic than the other bonding agents that contained Bis-GMA + UDMA + HEMA and Bis-GMA + HEMA [50]. In addition, it was demonstrated that a combination of Bis-GMA and HEMA was less cytotoxic than a combination of the following three substances: Bis-GMA, UDMA, and HEMA [12]. HEMA is the most frequent hydrophilic monomer present in adhesive systems. It is a low molecular weight monomer, which improves wettability and diffusion, and has penetration properties [60]. HEMA has been shown to diffuse rapidly across the dentine toward the pulp which, in turn, could induce hypersensitivity reactions in susceptible individuals [61]. Furthermore, HEMA may suppress the growth of many cell types and, in particular, induce a delay in primary fibroblast cell cycle progression by increasing the formation of reactive oxygen species (ROS) [56, 62, 63]. Various studies have reported different 50% toxicity (TC 50) values of HEMA. This difference has been suggested to be related to different sensitivities between different cell types [61]. Ratanasathien et al. [60] showed that the concentration of HEMA, which caused 50% inhibition of cellular metabolism after 24 h in contact with mouse fibroblasts, was 3.6 mmol l^{-1}. However, after 72 h, the TC 50 dropped to 1.0 mmol l^{-1}. Extrapolating this *in vitro* result to clinical situations, it could be speculated that the adhesive resin might promote more intense pulp damage over time. The cytotoxic effects of HEMA on human fibroblasts have identified the concentrations that cause TC 50 when compared with controls [64]. A 50% toxicity concentration of HEMA ranging from 10 μmol/l to 10 mmol/l has been demonstrated, mainly by mitochondrial dehydrogenase activity (MTT assay) and lactate dehydrogenase activity (LDH assay). It is well known that HEMA causes cell death by activating apoptosis [36, 55]; it interferes with the cell cycle and DNA synthesis, increases the production of reactive oxygen species, and induces a strong depletion of intracellular glutathione level even after very short times of exposure [24]. Di-methacrylates, Bis-GMA, UDMA, and TEGDMA are the most frequently used cross-linkers in adhesive systems. Some controversy exists about the biocompatibility of these monomers. Bis-GMA is considered cytotoxic with an ability to alter the cell cycle and induce oxidative stress leading to apoptosis and necrosis in a concentration-dependent manner [65]. In study conducted by Chang et al. [65], the concentration of 0.1 mM Bis-GMA reduced the metabolism of pulp cells by 50%. The determinant factor of the death was the monomer concentration, given that low concentrations of Bis-GMA induced apoptosis, whereas high concentrations induced death by necrosis or late apoptosis. Bis-GMA dissolves easily and may cause irritation or a lichenoid reaction of the oral mucosa in close and prolonged contact with gingival tissues, especially after cervical filling procedures [12, 66]. TEGDMA is a copolymer used in major recent biomaterials with resin phase as a diluent. *In vitro* studies have shown that TEGDMA induced toxicity in human fibroblasts associated with early and drastic glutathione depletion with subsequent production of ROS [14, 67], delayed cell proliferation, and mineralization processes. The cellular mechanisms underlying these phenomena remain poorly understood [14]. Experiments with several antioxidants suggested that ROS production is a major cause of TEGDMA-induced cellular cell death. TEGDMA is also known to be more cytotoxic to fibroblasts than HEMA [68]. UDMA provides improved compatibility with water and adhesives, which increases the

stability, making it behave similarly in both wet and dry dentins. UDMA released from dental materials was reported to reach a toxic level in human oral cells to induce their necrosis [69]. UDMA also induced morphological changes of pulp cells and decreased cell viability by 29–49% at concentrations of 0.1–0.35 mM [70].

Also, initiators may be released from dental adhesives and have been associated with cytotoxicity, related to their ability to generate free radicals [71]. The cytotoxicity of dentine adhesives may as well be attributed to camphorquinone (CQ), the most commonly used photoactivator [15, 56, 62]. For concentrations higher than 1 mM, CQ caused a significant concentration-dependent increase of intracellular ROS in human pulp fibroblasts (HPF) within 90 min of exposure. The cytotoxicity by CQ can be partly explained by its induction of cell cycle arrest and apoptosis. CQ also inhibited the expression of type I collagen, a major extracellular protein of dental pulp, suggesting the effect of CQ on matrix turnover and pulpal repair. CQ also stimulates prostaglandin E2 (PGE2) and PGF2α production of pulp cells [72]. It has been also documented that the camphorquinone acts not only as a cytotoxic agent but also as a mutagen, and its lixiviation may partly explain why these kinds of resinous products are considered as toxic agents [57]. Another substance known for its toxic, allergenic, and mutagenic effects and present in some dental adhesives is glutaraldehyde, which seems to be harmful by direct contact with mucous tissue, as well as by inhalation of its evaporated form [16].

A further factor directly related to the biological responses to adhesive systems concerns monomer conversion into polymers, obtained by the polymerization technique. It has been reported that the type of light curing unit and light parameters such as light spectrum and intensity affect the cytotoxic properties of dental adhesives, as well as polymerization time and application mode [66, 73]. All these parameters influence directly the degree of monomer conversion and consequently the release of residual monomers. Ye et al. [74] evaluated the effect of light irradiance and source on the photopolymerization of three commercial dental adhesives by monitoring the double bond conversion as function of time during and after irradiation. These authors observed that the time for Single Bond with little solvent to reach the conversion plateau was about 20 s, while the time for OneUp Bond F was about 25 s and for Adper Prompt as long as 40 s, and they indicated that the time required to reach the conversion plateau for adhesive polymerization is a valuable information for the dental clinicians. According to Peutzfeld [75], only 60% of monomers are completely bound in polymerized composite resin. Incomplete conversion results in an increased amount of residual monomer and creates the risk of leaching into the surrounding environment. However, even with sufficient light intensity, a certain amount of unreacted resin monomers may be released, which has a potential impact on the toxicity of the materials [56]. Adhesive/bonding agents cured with light-emitting diode (LED) units demonstrated higher cell survival rates in comparison with those cured with a halogen lamp [73]. Polymerization time can also influence on the cytotoxicity of adhesive systems. For resin-based dental materials, a shorter light-curing duration usually results in a lower degree of conversion, inferior mechanical properties, and a higher cytotoxicity. There is a dramatic difference in the responses of cells to the three conditions of polymerization (light curing for 0, 10, or 40 s) [35].

Cell culture assays provide a convenient, controllable, and repeatable method to assess the biocompatibility of materials. Increasing public concern regarding the use of animals in biocompatibility evaluation of dental materials has made *in vitro* testing more reasonable and ethically more acceptable [76]. In several studies, gingival fibroblasts and dental pulp fibroblasts were chosen as target cells to test the toxic effects of the dental adhesives. This choice was mainly due to the fact that under clinical conditions, gingival fibroblasts and dental pulp fibroblasts seem to be the most exposed cell type to dental adhesives. Pulp fibroblasts may be particularly affected by using dental adhesives on deep tooth cavities or when used as capping material, while the exposition of gingival fibroblasts to the adhesive layer may be more important by treating cavities with margins close to the gingiva, for example in restorations I and V class [77]. When testing is conducted in cell cultures, the monomers appeared to be less toxic in the presence of dentine and adsorption barrier; even the smaller monomer molecules diffuse through the dentine against an externally oriented fluid flow [39, 78]. According to Schmalz et al. [79], the dentine barrier test system for toxicity tests might mimic clinical *in vivo* oral environment and test the ability of the material to diffuse through dentinal tubules, which is better than direct cell material contact *in vitro* methods, and it has the potential to, at least partially, replace animal experimentation. Thus, it is ideal for testing cytotoxic effects of the material on dental pulp cells [25, 80]. One group of investigator show that low pH dentine-bonding agents have no effect on pulp-derived three-dimensional cell cultures when a 0.5 mm dentine barrier was placed between material and cells [81]. Under 0.3 ml/h perfusion conditions, the material with the lowest pH even increased the enzyme activity of the cell cultures. In contrast to them, another group of researchers has shown that components of the dentine-bonding systems may be capable of causing cellular damage, even when an interposing layer of dentine separates the material from the pulp. Therefore, they recommend to clinicians to consider applying a lining agent to the depths of their cavity preparations before applying a resin bonding agent [14]. Studies regarding the cytotoxicity of adhesives revealed potentially harmful effects on the gingival cells and may lead to mild to severe inflammatory reactions, cell changes, and cytotoxicity [77, 82]. Since self-etch adhesives are not fully dispersed during bonding process and cavity restoration with composite resin, there is concern about their contact time with the gingiva. Same authors suggested that contact with uncured primers and adhesives should be minimized. In this regard, long exposure of the acidic part of an aggressive self-etch adhesive without rinsing during its application, along with the chemical composition, might affect gingival fibroblasts [58, 83]. Dentists should follow the rules of adhesive application, precisely dose them, and not allow direct contact with the gums as, even after polymerization, adhesive agents exhibit potential cytotoxic activity [20].

5. *In vivo* biocompatibility testings of dental adhesives

The material is considered biocompatible when the interaction among a host, a material, and an expected function of material is in harmony. Dental material, furthermore, should not contain any toxic, leachable, and diffusible substances that could be absorbed and cause systemic responses such as teratogenic or carcinogenic effects. The ability of a material to even

enter into interaction with biological tissue and create a stable connection is essential to verify its biocompatibility [84].

In addition to already mentioned *in vitro* assays for testing biocompatibility of dental adhesives as well as of other dental materials, numerous *in vivo* assays were also used. Non-specific tissue reactions caused by dental materials *in vivo* are usually investigated by histological studies based on implantation of the test material in the different tissues of animals. They are usually used in mammals, but many other kinds of animals have been also used. The tested material can be injected directly or implanted (either directly or in the Teflon, silicone, or polyethylene tubes) in the different tissues determined by the use of the material, such as subcutaneous connective tissue, muscles, or bones [9]. Even the quality and specificity of the data obtained by *in vivo* models are questioned, because the used biological system cannot reproduce the same response as the target organ. However, the results are still valuable and useful. Thus, Santos et al. [85] were evaluating the biocompatibility of orthodontic adhesives in rat subcutaneous tissue. In that study, it has been detected the reduction of the inflammatory response at the end of day 15 compared to day 7 for almost all the tested adhesives.

There are conflicting results concerning direct pulp capping trails with adhesives on animals. Therefore, Watts and Peterson [86] suggested that biocompatibility studies and histological evaluation of the pulp healing after pulp capping need to be performed for each species in particular. So far, investigations were implemented on monkeys, dogs, rats, and cats. Cox et al. [87] showed in the monkey tooth model that most adhesive systems are biologically compatible with pulp, allowing pulpal wound healing and reparative dentine formation. Study of Nowicka et al. [88] analysed the pulpo-dentine response after direct capping with self-etch adhesive system. In the majority of the specimens, it was presented inflammatory pulp response with tissue disorganization and lack of dentinal bridge formation. Contrary to this, the teeth in the control group, capped with $Ca(OH)_2$, showed significantly smaller inflammatory pulp response with tissue disorganization and a considerably higher incidence of reparative dentine formation. These results are also in correspondence with those reported from the study of dogs from Koliniotou-Koumpia and Tziafas [89] and da Silva et al. [8]. They reported a total absence of hard tissue formation after direct pulp capping with dentine adhesive system and presence of chronic inflammatory cell infiltrate, giant cells, and macrophages around the resin globes, morphological cell alteration, and hyalinization of the pulp cells. The pulpal responses to the dentine bonding systems used in the mentioned investigations could be attributed mainly to their acidic nature. *In vivo* studies on primates' teeth showed normal healing of the pulp and also normal differentiation of the pulp cells. One of the reasons for such differentiates in results regarding pulp healing between human and animal teeth might be in different healing capacities among them. Generally, the animals are more resilient and adaptable to various external impacts and noxes around them in their evolutionary path. White et al. [90] in their study on rhesus monkeys in 1994 showed that there were no differences in cytotoxicity when the adhesives were set to dry or wet previously etched dry or wet dentine. Namely, in both cases, it has been observed that there was no inflammation of the pulp and that there has been a creation of reparative dentine beneath the remaining thin layer of dentine.

As opposed to animal studies, there are also clinical studies on humans, which are necessary for determining long-term biocompatibility of dental materials. The findings from above mentored studies on animals were similar to those obtained in study in human teeth of Cui et al. [91]. Namely, more pronounced histological changes in pulpal tissue in teeth treated with self-etching adhesive systems were found than in control group treated with $Ca(OH)_2$. Similar results were obtained also by Preira and associates [10]. In their study, the adhesive systems did not stimulate the dental bridge formation even after over 200 days after the pulp capping. On the contrary, the dental bridge healing was observed after 60 days in control group of pulp capping with $Ca(OH)_2$.

There are lots of opposite opinions of various authors about adhesives. Some authors consider that adhesives are safe, accurately biocompatible, and could be used for direct pulp capping on humans [92–94], whereas other researchers consider that adhesives are not suitable for direct pulp capping because of inducing the constant inflammation that does not heal [95–100]. Gwinnett and Tay [96] observed the pulp response after application of dentine adhesive to etched, deep, and unexposed coronal dentine, and they detected chronic inflammation of the pulp caused by adhesive in dental tubules. These so-called globules of resin in the tubules stimulate an immune reaction to a foreign body and are characterized by the appearance of mononuclear cell infiltration of macrophages or multinuclear giant cells. There has been also observed a degradation and loss of odontoblasts when the remaining dentine thickness was less than 0.3 mm. Furthermore, Hebling et al. [97] have found a hyaline alteration of extracellular matrix associated with oedema and hydropic pulp cell changes. Avles and Sobral [17] evaluated the biocompatibility of an etch-and-rinse adhesive system based on tertiary butanol applied in deep cavity human teeth with approximately 1 mm of remaining dentine by observing histological changes of the pulp tissue of humans at intervals of 1, 7, 14, and 21 days. They observed mild inflammatory infiltrate, preserved pulp tissue morphology, disorganization of the odontoblast layer in most specimens, as well as absence of bacteria at the intervals of 1, 7, 14, and 21 days.

Types of polymerization units also could be affected on the final cytotoxicity of adhesive system. Spagnuolo et al. [98] noticed that after lighting with LED units, there was a greater release of reactive oxygen that decreases cell survival than after lighting with halogen units.

There are a lot of studies that show that failure in the pulp capping is attributed to mistakes during operation, for example inadequate haemorrhage, an incomplete polymerization of the material, or usage of poor material properties. It is also known that a blood clot lyses could launch a series of chemotactic peptides that have a role in inflammation process [99]. Recent studies on human teeth have shown irreversible damage of the pulp and odontoblasts, and also chronic inflammatory response with macrophages and mononuclear giant cell infiltration observed over a period of 300 days following restorative procedure. The appearance of these signs indicates the typical immune response to the foreign substance in the organism. This is a result of interaction of resin and its components with plasma proteins, tissues, and connective tissue cells [100, 101]. In the following studies, it has been shown that non-polymerized resin compounds released from dental adhesives cause chronic pulp inflammatory response [102]. The response of the pulp to the dental materials could be modified by bacteria's and their

products, and also affect by carious dentine, cavity preparation, and bleeding. The reparative capacity of damaged pulp in those cases is already weaker, and immunological responses are more drastic; therefore, they might contribute to the cytotoxic effects of dentine-bonding agents [101].

Composite fillings and adhesives contain numerous components that could be released into the environment and cause a biological activity in the organism (cytotoxicity, carcinogenicity, mutagenicity, genotoxicity) [53, 103]. The monomers might be released in the saliva and diffuse into the gingiva, mucosa, salivary glands, and blood where their cytotoxicity or teratogenesis could contribute to the tumour formation which *in vivo* could be detected only after long-term interval. It is well known that high concentrations of UBMA, bis-GMA, TEGDMA, and other similar monomers have an inhibitory effect on the pulp cells. This effect results in chemically induced immunosuppression of the T-cells in the pulp. Thus, the pulp becomes more exposed, vulnerable, and susceptible to the bacterial infection. Acidic and non-acidic components of unpolymerized parts of self-etching dentine-bonding agents are responsible for the cytopathic effects on the odontoblasts and odontoblast-like cells. In an aqueous medium, adhesives dissolve even after polymerization and release components that could cause various effects on organism, such as allergic reactions (contact dermatitis and urticarial), systemic toxicity, cytotoxicity, and mutagenesis [104]. Once again, it is of crucial importance to mention the significance of the remaining dentine thickness [105]. Since the remaining dentine layer is thinner, thus more permeable, the cytotoxicity of applied material increases, and the response of the dentine-pulp complex is more intense [17]. High permeability dentine allows greater penetration of dentine-bonding agent and its components [39]. The highest cytotoxicity was observed in the early period of application, in the first 24–48 h after polymerization [106, 107].

According to these results, there are lots of concerns about pulp therapy with adhesive systems. Also, the results obtained from animal models cannot be extrapolated to human clinical condition.

Most practitioners purchase materials that are commercially available without any concerns about their biocompatibility. Today, despite the fact that clinicians use an increasing number of materials, most relevant studies concentrate on their physical properties with less emphasis on biological compatibility. There is a large gap between the results published by research laboratories and clinical reports. In order to assess the biocompatibility, clinical studies, as well as *in vitro* studies, are needed [108].

Toxicity of adhesive systems is associated primarily with short-term release occurring during setup time of material, due to insufficient polymerization, but also as long-term release of leachable substances generated by degradation over time [80]. Numerous studies showed that the differential toxicity of the adhesives could be attributed to the different ingredients, the interactions between them, and the degree of resin polymerization. Many studies of applied adhesive systems in direct contact with animal pulp tissue have supported the concept that bacteria, rather than dental materials, promote irritation of pulp tissue [87]. On the contrary, in other studies, it has been shown that those resin-based materials do not seem to be appropriate for use as pulp capping material and that no adhesive system should be applied directly on the pulp tissue [10, 35, 101, 109]. Thus, an adequate dentine barrier prevents or even reduces

the amount of monomers and other components capable of causing damage to the pulp-dentine complex [6, 110].

6. Conclusion

Biocompatibility is one of the most important properties of dental materials, and adhesives are no exception. Future innovation in adhesive systems should seek novel properties like tissue tolerance. Adhesives should be biofunctional, protective, and preventive, with health-promoting effects that contribute to a better prognosis for restorative treatments and its biocompatibility.

According to results obtained from our previous *in vitro* studies, we can affirm that tested adhesives did not have genotoxic potential. *In vitro* studies are likely to differ from *in vivo* condition because higher concentrations of tested materials are usually used *in vitro* compared to the concentrations that may be used during regular handling with dental materials. Furthermore, *in vivo* studies present numerous factors such as saliva, mucus layer, creatine levels, blood flow, and oral flora that can influence the oral cavity protection. Consequently, we can conclude that commercial available dental adhesives, whether they are used alone or in combination with dental composites, are safe for clinical use.

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