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DISCUSSION PAPER

In vitro and in vivo studies on the toxicity of dental resin components: a review

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Abstract In vitro and in vivo studies have clearly identified that some components of restorative composite resins, adhesives, and resin-modified glass ionomer cements are toxic. The mechanisms of cytotoxicity are related firstly to the short-term release of free monomers occurring during the monomer–polymer conversion. Secondly, long-term release of leachable substances is generated by erosion and degradation over time. In addition, ion release and proliferation of bacteria located at the interface between the restorative material and dental tissues are also implicated in the tissue response. Molecular mechanisms involve glutathione depletion and reactive oxygen species (ROS) production as key factors leading to pulp or gingival cell apoptosis. Experimental animal approaches substantiate the occurrence of allergic reactions. There is a large gap between the results published by research laboratories and clinical reports.

Keywords Composite resin · Resin-modified glass ionomer cement · Adhesive · Free monomers · HEMA · TEGDMA · Free radicals · Cytotoxicity · Glutathione · Allergy · Mutagenicity

Do dental materials fulfill the clinical requirements?

Out of the 290-million cavities restored each year in the United States, 200 million are replaced for failed restorations [4]; the use of amalgam as restorative material is decreasing, whereas the number of tooth-colored composites and resin-modified glass ionomer cements (RM-GIC) restorations is increasing. Despite dentistry becoming gradually a sophisticated biomedical discipline, one of its Achilles' heels seems to be the restorative materials that are responsible for such an unacceptable high proportion of failures. Despite the fact that composites and RM-GIC have improved their physico-chemical properties, their intrinsic toxicity remains high, at least when they are evaluated in vitro. In addition, due to the fact they are tooth-colored, dental practitioners use less other restorative materials such as silver amalgam. Secondary caries is the most frequent reason for replacing restorations. A clear-cut explanation for that is that resins shrink during polymerization, leaving a gap colonized by bacteria between the material and the cavity walls, and the so-called hybrid layer does not exclude bacterial penetration [15]. This contributes to the development of secondary caries. In addition, unbound free monomers are excellent substrates for cariogenic bacteria [32]. Other reasons for failures are either mechanical, hypersensitivity, or both, and a few teeth can only be cured by endodontic treatment [9, 49]. Altogether, there is a need for the reevaluation of the physicochemical and biological properties of materials currently used in restorative dentistry that apparently do not fulfill the requirements of safety and longevity.

During the last few decades, the enthusiasm was great for resin composite and resin-modified glass ionomer cements that are tooth-colored materials considered as possible substitutes to mercury-containing silver amalgam filling. Ecological and esthetic considerations lead to the decrease of a material with long standing clinical experience to the profit of resin-containing materials with a partially very short “market life span” (only months sometimes). It is clear that defective mechanical properties

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such as shrinkage due to dimensional changes during resin polymerization and poor resistance to abrasion have decreased the initial eagerness.

Three comments should be done. Firstly, unbound monomers favor bacteria proliferation, especially the microorganisms implicated in caries formation. Therefore, they contribute to the development of secondary caries. Secondly, cellular and molecular mechanisms of cytotoxicity are initiated by the monomers, leading to pulp alteration and retraction of the gingival margin [29, 57]. Thirdly, local and systemic allergic-related reactions have been reported [34]. In this context, between 0.7% and 2% of patients and dental practitioners display allergic reactions [10, 35, 50, 55].

A number of in vitro and animal studies clarify the mechanisms of adverse reactions. In contrast, there is a need for more well-controlled unbiased clinical reports. Either there is no harmful reaction or they are not reported. In any case, there is an enormous gap between in vitro and in vivo studies. Apparently, the follow-up of restorative materials do not provide information on the actual physiopathological status of the pulp, probably because the methods used by clinicians are rather crude and not accurate enough. The reasons for the discrepancies between the two groups of reports are not yet clear. The link between the work going on in laboratories and the restorative therapies is missing up to now. However, it would be surprising that the harmful effects that are well identified in laboratory studies, and reviewed in this paper, have no clinical consequences. In the next future, we might face major problems in terms of public health. In the present article, in vitro data and in vivo animal studies will review the adverse effects of resin composites, RM-GIC, and dental adhesives.

Composition of composite resins and resin-modified GIC

Composite resins are tooth-colored filling materials composed by a mixture of (1) an organic polymerizable matrix, (2) particulate ceramic reinforcing fillers, (3) molecules promoting or modifying the polymerization reaction, and (4) silane coupling agent connecting the fillers and the organic matrix. The filler is inorganic in nature, formed by quartz, borosilicate, lithium–aluminum–silicate glasses, and amorphous silica. The terms of "all-purpose" or "universal", "anterior" or "posterior" describe the intended use of composites by dental practitioners. They are better than "conventional" or "traditional", "microfilled" or "hybrid", "flowable" or "packable", which were used formerly. This recent terminology does not refer to the particle filler size and is inappropriate for the characterization of the type of material, considerable variations of size, shape, and volume of the filler occurring within and between materials. In addition, fillers do not seem to play a major role in the biocompatibility of the material. The organic polymerized matrix seems to be responsible for most of the reported undesirable effects.

Many in vitro studies have shown that the polymerization reaction that produces the cross-linked polymer matrix from the dimethacrylate resin monomer is never complete and adverse reactions are due to the release of nonpolymerized monomers such as triethylene glycol methacrylate (TEG DMA) or 2-hydroxy-ethyl-methacrylate (HEMA). Unbound free monomers seem to be directly responsible for the cytotoxicity of resin composites on pulp and gingival cells, and they are probably also implicated in the allergic potential of the material [19]. Recent progress of dental composites did not change the occurrence of cytotoxic effects, and in this context, similar toxic levels were obtained with packable and nonpackable dental composites [22].

Conventional glass ionomer cements (GIC) are composed of glass, e.g., Ca–Al–F–silicate–glass, and poly (acrylic) carbonic acids. They have a good biocompatibility [29]. Along this line, rat first maxillary molars were submitted to cavity preparation and filled with Fuji IX GIC. After 8 days, a moderate inflammatory reaction was seen in the pulp, which was totally resolved at day 30 [72]. However, to improve mechanical properties and facilitate clinical handling, resin-modified glass ionomer cements (RM-GIC) have been developed by the industry. RM-GICs contain poly(acrylic)acids, photocuring monomers (HEMA) or a photocuring side chain grafted onto the poly(acrylic) acid and an ion-leaching glass. GICs have been also combined with resin composite components, such as dimethacrylates, benzophenone, and camphoroquinone, giving rise to a group of mixed restorative materials called compomers and RM-GICs. These materials and dentin bonding agents have now been studied and shown to be cytotoxic for pulp and gingival cells [58, 78].

Mechanisms of cytotoxicity

Although unbound free monomers released by dental resins during polymerization and later are considered by a majority of authors to be responsible for the cytotoxic effects, additional mechanisms have been also proposed.

Short-term release of free monomers occurring during the monomer–polymer conversion

Unbound monomers and/or additives are eluted by solvents or polymer degradation within the first hours after initial polymerization. The release is due to defective photopolymerization, thermal, mechanical or chemical factors. Approximately 15–50% of the methacrylic groups remain unreacted [19]. Due to the efforts of the industry, the

percentage of unbound monomers has been decreased during the past 10 years, but the problem is still not eradicated. Up to now, there is no total conversion during polymerization. It may be expected that at the end of initial polymerization, most of the monomers will react with the polymer network and the quantity of residual monomers is less than a tenth of the remaining methacrylic groups, which, therefore, have been evaluated as no more than 1.5–5%. However, this is enough to contribute to major cytotoxic effects [75]. The reaction is also dependent on dentin permeability and residual dentin thickness [8]. The residual dentin layer absorbs unbound monomers and, therefore, contributes to the decrease in the cytotoxicity of the material, but this parameter is not directly under the control of the dental surgeon, although the formation of reactionary dentin may be stimulated by preparative steps. Dentin permeability may also be modified by calcium/phosphate precipitation in the lumen of the tubules leading to sclerotic dentin formation. It has also been shown that the surface of composite resins exposed to oxygen during curing produces a nonpolymerized surface layer containing formaldehyde, which by itself is an additional factor of cell toxicity [64].

Monomers have been identified in dental composites eluates by gas and liquid chromatography/mass spectrometry. A considerable concentration of the comonomer triethyleneglycol-dimethacrylate (TEGDMA) and minor concentrations of the basic monomers bisphenol-A glycidyldimethacrylates (Bis-GMA) and UDMA and the comonomer HDDMA have been detected with these methods [28, 74]. The comonomer TEGDMA and the photostabilizer 2-hydro-4-methoxybenzophenone (HMBP) are cytotoxic and inhibit cell growth, but the most crucial consequence is that the intracellular glutathione level is decreased to 85±15% by TEGDMA [17, 18, 75–77].

In vitro evaluation of the cytotoxicity of 35 dental resin composite monomers/additives indicates moderate to severe cytotoxic effects [28]. The results vary according to the material tested, but also they are strongly dependent on the cells used for testing. For example, human periodontal ligament and pulp fibroblasts are more sensitive than 3T3 and gingival fibroblasts [28]. With the exception of very few reports, there is a general consensus that resin-containing restorative materials are cytotoxic [25, 26, 64], especially after mixing.

At early intervals, resin-containing materials are more cytotoxic than at later intervals. However, long-term effects should also be taken into consideration.

Leachable substances generated by erosion and degradation over time

The chemical characteristics of leachable substances determine the diffusion through the polymer network. Leachable components are released due to degradation or erosion over time. Chemical degradation is caused by hydrolysis or enzymatic catalysis. Unspecific esterases and human saliva-derived esterase may readily catalyze the biodegradation of commercial resinous materials [24, 26, 38]. Interactions between resin monomers and commercial composite resins with human saliva-derived esterases and pseudocholinesterase occur in the oral cavity and they contribute to the degradation of composite resins. Incubated in vitro with cholesterol esterase for 8, 16, and 32 days, resin composites release 2,2-bis [4(2,3-hydroxypropoxy)-phenyl]propane (bis-HPPP) and TEGMA. Depending on the material (lower filler vs higher filler composite), and consequently, on the matrix/filler ratio, lower or higher amounts of bis-HPPP and TEGMA are produced between 0 and 8 days [71].

Water or other solvents enter the polymer, leading to the release of biodegradation products, namely, oligomers and monomers. This form of erosion leads to weight loss of the polymer. Softening of the Bis-GMA matrix allows the solvents to penetrate more easily and expand the polymer network, a process that facilitates the long-term diffusion of unbound monomers [20, 21].

Release of ions

Resins and RM-GIC release ions such as fluoride, strontium, and aluminum. Some ions, such as fluoride, are expected to be beneficial and reduce the development of secondary caries. Presumably, the fluoride content of toothpastes and nutriments reload the material, so that the resins or RM-GICs do not become porous. Other ions are implicated in the color of the restorative material, and these metal elements may interfere with the biocompatibility of the resin because they are implicated in the Fenton reaction producing reactive oxygen species (ROS) that are cytotoxic. The concentration of F^{-} and Sr^{2+} is too low to be cytotoxic. In contrast, Cu^{2+}, Al^{3+}, and Fe^{2+} are present in toxic concentrations. The cytotoxic cascade was shown to be enhanced by metals, such as aluminum and iron, present in various amounts in composite resins and RM-GIC [75–77].

The role of bacteria at the interface between the restorative material and dental tissues

The presence, and consequently, the effects of bacteria located at the interface between the resin and the dental tissues probably constitute important factors [32]. EGDMA and TEDGMA promote the proliferation of cariogenic microorganisms such as *Lactobacillus acidophilus* and *Streptococcus sobrinus*. As a confirmation, TEDGMA stimulates the growth of *S. mutans* and *S. salivarius* in a pH-dependent manner [42]. This provides an explanation for the secondary caries lesions that develop beneath resin-

containing restorative materials. In addition, bacterial exotoxins have noxious effects on pulp cells after diffusion throughout dentin tubules.

For years, some publications refuted any direct cytotoxic effects of the resin monomers upon the dental pulp and laid the blame on bacterial contamination [5, 6]. This is still a matter of controversy and a few reports still consider that the pulp reaction to adhesive systems is generally minimal [51, 52]. They incriminate the colonization by bacteria of the wide interface between the composite (or adhesive) and cavity walls. Bacteria may produce acids that can be responsible for the pulp reaction [5, 6]. This was considered true in the early 1980s when important volume shrinkage followed chemo-polymerization and produced gaps wider than 10 μm [6]. This interval was decreased by the layer-by-layer photopolymerization technology. Improvements of resin-containing materials have reduced the shrinkage. New adhesive technologies lead to the formation of a hybrid layer and diminish the interface to less than 1 μm [33]. However, this is still a large gap for many microorganisms such as lactobacilli that are less than 0.1 μm in diameter, and therefore, the microbial parameter cannot be ignored. Some authors have emphasized the importance of hemorrhage control and its interference with bacterial contamination [13]. However, the major issues today seem to be the short-term and long-term release of unbound toxic free monomers [25, 26, 75] rather than the release of acids and toxins by bacteria.

Molecular mechanisms of cytotoxicity: recent data

For more than 10 years, it is known that resin composites, dentin bonding components, and RM-GIC are cytotoxic. HEMA, TEGDMA, and UDMA have been incriminated [58]. Lefebvre et al. [46] have analyzed the action of TEGDMA, dimethylaminoethyl I metacrylate (DMAEFMA) on oral epithelial cells. It inhibits cell growth and total polar lipid synthesis. Even after polymerization, dental resins may elute into the immediate environment unbound monomers that alter various cell metabolic processes [27, 48, 75–77]. Other biological parameters have been studied, but their importance still needs to be established. Along these lines, HEMA and TEGDMA suppress heat shock protein 72 (HSP 72) expression in human monocytes [53]. TEGDMA induces mitochondrial damage [48]. The cytotoxicity of dentin-bonding resins seems also to be associated with defective intracellular tyrosine phosphorylation [39].

The exposure time and interactions between dentin-bonding components are important parameters depending on the type of cells studied. TEGDMA is about twofold to fivefold more toxic than HEMA for pulmonary cells [41]. Intracellular glutathione level is decreased by TEGDMA within the first 2–6 h after setting [18]. At a TEGDMA concentration of ≤2.5 mM, a regular reincrease is observed; whereas at higher concentrations, a continuous depletion occurs, concomitant with a significant decrease of cell viability.

HEMA is released from light-cured or RM-GIC and compomer cements [31]. Intracellular tyrosine phosphorylation is inhibited by HEMA and not by TEGDMA [39]. Chang et al. [11] have shown that HEMA induces cell growth inhibition and cycle perturbation; indeed, the arrest of cell S–G phases. The G_2/M and S phases arrest, glutathione depletion, and ROS production are key factors leading to cell apoptosis.

TEGDMA monomers released from dental resins induce in vitro a concentration-dependent and variable cytotoxic effect. Toxic concentration 50 (TC50) was obtained with 1.2±0.9 and 2.6±1.1 mM of TEGDMA for gingival and pulp fibroblasts, respectively. The depletion of glutathione due to the leaching of monomers starts at 15–30 min and is almost complete at 4–6 h. As a valuable demonstration of the importance of this mechanism, antioxidants such as Trolox, ascorbate, and *N*-acetylcysteine (NAC) prevent the TEGDMA-induced toxicity while GSH depletion is partially inhibited. Trolox, preventing the cell damages mediated by resin-containing dental restorative materials, inhibits the production of ROS that occurred after 3–4 h incubation in the presence of TEGDMA. Ascorbate increases, in a dose-dependent manner, the toxic effects of the eluates (additional 17–24% depletion of glutathione). D-mannitol neutralizes the toxic effect of ascorbate [70, 73, 76, 77]. The decreased toxicity of free monomers by these antioxidative agents paves the way for clinical attempts to reduce the potential noxious effects of resin-containing dental materials.

We recently reported that TEGDMA inhibits and potentiates glutathione transferase P1 (GSTP1) at high and low concentrations. Isoforms of GSTP1 have been identified in gingival fibroblasts, and depending on the phenotype, the reaction to TEGDMA varies. Cells of the GSTP1 *A/*A group and *A/*B variant show a weak inhibition and no significant difference between the two groups of cells. In contrast, the toxic dose 50 (TD50) value for *B/*B was significantly lower, and therefore, this isoform of GSTP1 may be less effective than *A/*A in detoxifying TEGDMA. These data suggest that GSTP1 polymorphism may be involved in the interindividual susceptibility to TEGDMA cytotoxicity [47]. Once confirmed on a greater scale, these data may contribute to the identification of a group of patients that are more susceptible than the rest of the population to present harmful reactions to resin monomers.

Local and general toxicity and mutagenic effects

In vitro studies

Cytotoxicity tests involve the evaluation on cell cultures of enzyme activities, membrane integrity, alteration of cell morphology, determination of cell growth inhibition, and determination of the effective dose that cause 50% reduction of cell proliferation (ED50). Counting surviving cells (TC50=50% reduction of cell survival), measurement of proliferation rates, synthesis of cellular macromolecules, and determination of enzyme activities are the most used and recognized indicators. A widely used method is the MTT test, although succinic dehydrogenase (SDI) and alkaline phosphatase responses have also been used [12]. Photopolymerized standardized cylinders of composite resin or RM-GIC, prepared according to the manufacturer's instructions, are incubated in culture medium. After 48 h of incubation, cells are incubated with or without (group control) the biomaterial eluates. Conventional and resin-modified GIC toxicity studies evaluated with the MTT test suggest that RM-GICs are more toxic than conventional GIC [45]. Depending on the cell lines used and the method of evaluation, the results may vary [80]. As an example, human pulp fibroblasts (not cloned) and human gingival fibroblasts tested with the MTT test and lactate dehydrogenase activity assay (LDH) gave different results, and MTT was more sensitive than LDH [36]. Despite methodological differences, altogether, the results underline the cytotoxic effects of the monomers released by resin-containing restorative materials.

TEGDMA is hydrophilic and interferes with oral tissues. The compound can penetrate membranes and reacts with intracellular molecules. Specifically, glutathione–TEGDMA adducts are formed, a mechanism reducing cellular detoxifying potency [27]. Significant toxic effects of TEGDMA and HEMA on glucogenesis were reported on isolated rat kidney tubules, although less toxic than the effects of mercuric chloride or methylmercury chloride [59]. Synergistic effects of H_2O_2 from dental bleaching compounds and monomers released by dental composites lead to increased toxicity on kidney cells [23]. Using the release of LDH as a biological indicator, toxicity was also reported in confluent alveolar epithelial lung cell lines in vitro [60].

Evaluation of the mutagenicity has shown that TEGDMA causes large DNA deletions in mammalian cells (genotoxicity) [68, 70]. TEGDMA, HEMA, and GMA induce an increase of the number of mutants by a factor of 2 to 8 [67] and the formation of micronuclei [69]. Using the Comet assay (alkaline single cell microgel electrophoresis assay), TEGDMA, UDMA, and Bis-GMA induce significant but minor enhancement of DNA migration, a possible sign for limited genotoxic effects [43]. More recent data give evidence of a possible risk factor for tumor initiation in human salivary glands [44].

In vivo experimental studies

Direct pulp capping with adhesive resin-based composite leads generally to infection and necrosis [1, 66]. However, this is still a matter of controversy, and a few authors still believe that a better sealing of the restoration will prevent bacteria contamination, and consequently, better healing of the exposed pulp [5, 52]. Mild to severe inflammatory response was observed in human pulps treated with Scotchbond Multi-purpose, and no mineralized tissue formation was detected [16]. The clearance, distribution, and elimination of TEGDMA has been extensively studied [61, 62]. About 4% of the ^{14}C-TEGDMA injected in the jugular vein of guinea pigs was found in different tissues such as muscle, kidney, skin, blood, and liver after 24 h, whereas 61.9% was exhaled. Exhalation seems to be the major route of elimination [61]. Gastric, intradermal, and intravenous administration of ^{14}C-TEGDMA establish that most is excreted in 1 day, and the peak equivalent TEGDMA level in mouse and guinea pig is 1,000-fold less than known toxic levels [62]. In a third publication, Reichl et al. [63] have shown that after the administration of the radioactive monomer either in the gastric tube or after intradermal injection, the uptake was almost completed within 1 day. Low fecal (<1%) and urine (about 15%) elimination was noted, whereas between 60% and 65% was exhaled. In addition, ^{14}C-pyruvate seems to be formed as a toxic ^{14}C-TEGDMA-intermediate. Confirming previous findings, despite the high doses administered in this experimentation, after 24 h, the doses found in tissues were 100,000-fold less than known toxic levels [63].

Studies in animals and humans have shown mild to severe inflammatory pulp reactions, leading in many cases to cell apoptosis, followed by severe pulp alteration [29].

Estrogenic effects

Estrogenic activity of three fissure sealants has been associated with bisphenol A dimethacrylate (BPA-DMA) rather than with Bis-GMA [79]. It was shown that BPA-DMA can be cleaved by unspecific esterases producing BPA, which then may elicit an estrogenic reaction in vitro [3, 65]. The total amount of TEGDMA released per tooth is in the order of 0.25 mg, most of the release occurring on the first day [30]. Adverse effects were denied by Nomura et al. [54], and in any case, the dose released is considered to be too low to have any consequence on patients [64]. Moving from in vitro to in vivo studies, leached components or bisphenol A

administered intragastrically daily to female mouse display 54.5% reduction in the number of pregnancies vs 100% in control mice. An effect on the ovaries was also reported, BPA having adverse effects on the fertility and reproductive system in female mice [2]. However, a study involving sealants applied to human subjects shows no detectable bisphenol A in the blood serum and saliva after 24 h [24].

Allergic responses to dental resins

The results from the Norwegian National Dental Biomaterial Adverse Reaction Unit listed for 4 years of activity from 1993 to 1997 indicate that out of the 296 patients who had been patch tested with substances in dental materials, 23% were positive to gold, 28% to nickel, 9% to palladium, 6% to mercury, and 8% to one or more components of resin-based materials [81]. Skin and mucosal reactions are associated with dental materials [35]. Skin symptom or hand eczema caused by contact allergy, appearing as an eczema, has been evaluated in a nonselected population of patients as 12%, in contrast with 27% found in a dentist group, 2% being caused by acrylic resin materials [34]. Another report indicates that 28% of the dentists have contact allergy to nickel, perfumes, and other chemicals with diagnosed hand eczema. Seven percent of the dentists display skin symptom to resin-based materials. Allergy to methacrylate of dental personnel is below 1% [55, 56, 82], but around 2% in another investigation [50].

According to Hume and Gerzina [35], dermatoses are the most frequently diagnosed adverse responses, together with paresthesia of the fingertips and allergic pharyngitis. HEMA causes contact dermatitis, and induces irritation and delayed hypersensitivity when applied to the skin of guinea pigs [40]. After the placement of resin composite restoration for orthodontic treatment, a few cases of allergic response have been published with edema and vesiculation of the oral mucosa and lips. Case reports refer to severe dermatitis on the limbs, trunk, and face whereas allergic reactions (bronchospasm, whole body urticaria, and blistering of the face, ears, and lips) are related to the placement then removal of sealants containing TEGDMA.

In the oral cavity, lichenoïd reactions have been observed in close relationship with composites and GIC [7, 36, 37]. It is also possible that immune cells play a role in the pulp response. Mast cell density is increased by adhesive resins and GIC [14].

Conclusions

It is clear that resin-containing restorative materials release unbound free monomers, immediately after setting and later. These monomers are cytotoxic in vitro for pulp and gingival cells. Leaching of some ions seems also to be implicated in cell alterations. Depletion of glutathione, production of ROS, and a few other molecular mechanisms have been identified as key factors leading to apoptosis and/or pulp necrosis. In addition, resin monomers stimulate the development of cariogenic bacteria at the interface between the material and the walls of the cavity. Dental resins have no intrinsic antibacterial properties, but some additives may have this effect. However, monomers provide a good substrate for cariogenic bacterial strains. This may lead to the formation of secondary caries, and further to the long-term degradation of the polymers, two factors involved in the failure of the restoration.

It is surprising to note that there is a large gap between the numerous reports on in vitro adverse reactions, what has been established on allergy and dermatitis, and the clinical evaluation of composites, adhesives, and RM-GIC [64]. In view of all the problems identified mostly in vitro, there is a need for clinical studies establishing or refuting a correlation with the in vivo situation.

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