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Epigallocatechin-3-Gallate Reduces Cytotoxic Effects Caused by Dental Monomers: A Hypothesis

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Resin monomers from dental composite materials leached due to incomplete polymerization or biodegradation may cause contact allergies and damage dental pulp. The cytotoxicity of dental resin monomers is due to a disturbance of intracellular redox equilibrium, characterized by an overproduction of reactive oxygen species (ROS) and depletion of reduced glutathione (GSH). Oxidative stress caused by dental resin monomers leads to the disturbance of vital cell functions and induction of cell apoptosis in affected cells. The nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway plays a key role in the cellular defense system against oxidative and electrophilic stress. Epigallocatechin-3-gallate (EGCG) can activate the Nrf2 pathway and induce expression of a multitude of antioxidants and phase II enzymes that can restore redox homeostasis. Therefore, here, we tested the hypothesis that EGCG-mediated protection against resin monomer cytotoxicity is mediated by activation of the Nrf2 pathway. This study will help to elucidate the mechanism of resin monomer cytotoxicity and provide information that will be helpful in improving the biocompatibility of dental resin materials.

MeSH Keywords: Cytotoxicity, Immunologic • Reactive Oxygen Species

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Background

Resin-based materials are now used ubiquitously in dentistry, and have provided a satisfying alternative for amalgam to restore traumatized and decayed teeth during recent decades. However, the biocompatibility of dentin bonding agents has recently attracted interest. It has been well documented that large amounts of residual resin monomers may leach from restorative in the first days after application due to insufficient monomer-polymer conversion [1,2]. Moreover, clinically polymerized composites are also susceptible to biodegradation by wear and enzyme activities in saliva, which may result in the release of monomers and subsequently expose oral cells and tissues to an environment with large amounts of monomers for a long period of time [3]. These monomers may have irritating effects on adjacent oral tissues and cause contact allergies, potentially including dental pulp if monomers are disseminated through dentinal tubules [4]. Actually, several principal monomers in dental composites have been identified as cytotoxins. Based on previous studies, the concentrations of leached methacrylate monomers can reach as high as millimol levels, which are far higher than their median lethal dose (LD50) values [5–9]. Thus, it is necessary to understand the exact mechanisms underlying the cytotoxicity and to find innovative strategies to decrease or eliminate their toxicities. Although the cytotoxicity mechanisms of resin monomers have not been delineated, prior reports have suggested that the cytotoxicity of resin monomers is related to disturbances in intracellular redox equilibrium due to induction of reactive oxygen species (ROS) concomitant with depletion of anti-oxidative glutathione (GSH) [10–12]. *In vitro* experiments involving multiple target cells have shown that resin monomers interfere specifically with various vital cellular functions, inducing apoptosis [13].

Based on the findings that oxidative stress is the main reason for the cytotoxic effects of resin monomers, Nrf2 has been studied. The nuclear factor-erythroid 2-related factor 2 (Nrf2) is a key cyto-protective transcription factor in this system, a so-called master regulator of genes responding to disruption of redox homeostasis [14]. Specifically, Nrf2 activates transcription of genes regulated by the antioxidant response element (ARE), including genes encoding phase II detoxifying and antioxidant enzymes responsible for protecting cells from electrophile toxicity and oxidative stress. Thus, Nrf2 is an attractive target for activation to decrease or eliminate resin monomer toxicities. Currently, an increasing number of natural compounds have been found to exert anti-inflammatory and antioxidant properties by involving the Nrf2-Keap1 signaling pathway [15,16]. Epigallocatechin-3-gallate (EGCG) is found in many edible plant components and an effective free radical scavenger. EGCG can regulate Nrf2 activity by freeing it from association with its suppressor Kelch-like ECH-associated protein 1 (Keap1) and inducing expression of genes encoding phase II enzymes [17]. Therefore, EGCG may represent a new approach for protecting cells from resin monomers. However, to the best of our knowledge, there are no available data regarding the relationship between EGCG and modulation of Nrf2-Keap1 in dental monomer-induced cytotoxicity. The aim of this review is to present interesting new evidence on the potential application of EGCG in protecting dental patients from resin monomer-induced cytotoxic effects.

The Hypothesis

Our hypothesis is that the Nrf2 pathway plays a key role in cyto-protection during resin monomer-mediated oxidative stress. Given the Nrf2 knockout mouse phenotype (hypersensitivity to oxidative insults, phase II detoxifying enzyme deficiency), we also investigated whether activation of Nrf2 decreases or eliminate resin-associated electrophilic and oxidative damage and whether EGCG-induced upregulation of the Nrf2 pathway attenuates dental monomer-induced electrophilic and oxidative damage.

Basis for the Hypothesis

Oxidative stress and dental monomer-induced cytotoxicity

There exists a highly sophisticated antioxidative system consisting of non-enzymatic and enzymatic elements to maintain a balanced intracellular redox homeostasis. Under physiological conditions, ROS are generated in mitochondria at low, manageable levels. Mitochondrion-derived ROS include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^-). Glutathione (GSH), the key component of the antioxidative defence system, functions to directly scavenge ROS, or by acting as a substrate for glutathione peroxidase (GPx), which catalyzes the reduction of H_2O_2. The enzymatic antioxidants directly control cellular redox homeostasis by regulating the levels of particular ROS. Superoxide dismutase (SOD) functionally catalyzes the conversion of O_2^- into O_2 and H_2O_2, whereas catalase (CAT) and GPx subsequently conduct the conversion of H_2O_2 to H_2O and O_2 [18,19]. However, stimulation with resin monomers results in increased levels of ROS [13]. Dental resin monomers can chemically react with GSH and lead to the depletion of the intracellular GSH pool. GSH is the primary antioxidant central to the regulation of cell response towards oxidative stress induced by dental monomers [20]. The subsequently elevated ROS, especially H_2O_2, is the secondary result of GSH depletion. Then, the activities of intracellular antioxidant enzymes are differentially affected by dental monomers, indicating the induction of oxidative stress. When the formation of ROS during monomer exposure is beyond the capacities of

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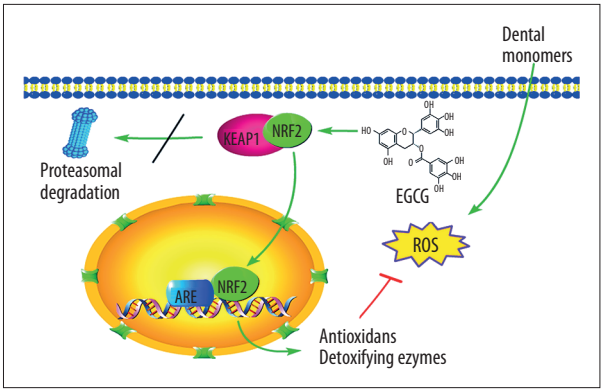

Figure 1. A schematic diagram summarizing how Epigallocatechin-3-gallate (EGCG) can eliminate dental resin monomer-associated oxidative damage. Dental resin monomers can lead to the over-production of ROS. EGCG helps Nrf2 escape from Keap1 and Nrf2 nucleus translocation, then activates the Nrf2 pathway and induces expression of antioxidants and phase II enzymes, which contributes to eliminating the over-produced ROS and detoxification of dental monomers cytotoxicity.

anti-oxidative mechanisms, the overproduced ROS can react with cellular macromolecules, such as lipids, proteins, and DNA. The interactions of ROS with DNA may damage DNA bases and cause lesions that can block progression of replication, which results in DSBs in the chromosome and activation of functional cell cycle checkpoints [10,21]. Accumulation of DSBs caused by HEMA triggers repair signal transduction pathways that establish cell-cycle arrest and activate programed cell death [22].

Nrf2 plays a key role in cellular antioxidant defense system

Nrf2 belongs to the "cap n' collar" family of transcription factors and is regarded as a master regulator of cytoprotective responses to oxidative and electrophilic stress [23,24]. Under basal conditions, Keap1 serves as a substrate adaptor protein for the CUL3-dependent ubiquitin E3 ligase complex. Nrf2 forms a "hinge-and-latch" complex with 2 Keap1 proteins [25,26]. Nrf2 is kept transcriptionally inactive when bound to Keap1. Nrf2-bound Keap1 forms a functional ubiquitin E3 ligase complex when it interacts with Cullin 3 (CUL3). This ubiquitin E3 ligase complex polyubiquitinates Nrf2 rapidly, resulting in suppression of the transcriptional activity of Nrf2 and Nrf2 degradation.

When cells are exposed to oxidative or electrophilic stress, modification of cysteine residues in Keap1 induces Nrf2 dissociation from Keap1 and subsequent nucleus translocation of Nrf2. Consequently, Nrf2 accumulates in the nucleus, where it induces the expression of its ARE-regulated target genes, including the genes that encode phase II detoxifying and antioxidant proteins (Figure 1). The phase II is an important metabolism process of xenobiotics in which the activated forms of xenobiotics are enzymatically catalyzed and transformed to larger, less active forms that can be excreted more easily. A summary of phase II conjugation enzymes and their actions is presented in Table 1 [27]. Compared with wild-type mice, Nrf2 knockout (*Nrf2–/–*) mice have much lower mRNA and protein levels of detoxifying enzymes and, consequently, are extremely susceptible to oxidative stress [28].

EGCG can eliminate oxidative damage by activating Nrf2 pathway

EGCG is the most abundant in green tea, with both anti-inflammatory and antioxidant properties. The antioxidant properties of EGCG may be attributed to 3 aspects. First, oxidized EGCG reacts with GSH to form conjugates, leading lower cellular GSH levels, which in turn triggers phosphorylation of Nrf2. Second, the reactive forms of EGCG may also interact directly with the highly reactive cysteine residues of Keap1, favoring release of Nrf2. Third, EGCG auto-oxidation produces ROS, which stimulates Nrf2 phosphorylation, further favoring Nrf2 nuclear translocation [29]. EGCG treatments have been shown to increase the nuclear distribution of Nrf2 markedly and upregulate NRF2-target genes including heme oxygenase-1 (HO-1), NADH quinone oxidoreductase 1 (NQO-1), GST, and those involved in GSH and thioredoxin systems in Kunming mice4 [30]. EGCG derived from green tea has also been reported to activate MAPK pathways (ERK, JNK, and p38) through ARE-regulated genes encoding for phase II antioxidants and detoxifying enzymes [17]. Additionally, EGCG has been shown to protect endothelial cells against polychlorinated biphenyl-induced cell damage, and these protective effects are associated with dose-dependent upregulation of the expression of Nrf2-controlled antioxidant genes, including NQO1and GST [31].

Evaluation of the Hypothesis – Dental Monomer-Induced Cytotoxic Effects and Nrf2 Pathway

Recently, some studies have found a relationship between the cytotoxic effects of dental monomers and Nrf2 expression. Takahiro et al. reported that methyl methacrylate (MMA) increases promoter activity at the GST alpha 1 gene (GSTA1) in a dose-dependent manner through the ARE, resulting in upregulated expression of GSTA1 [32]. The same group demonstrated that HEMA can induce overexpression of Nrf2 and Keap1 in HepG2 cells, as well as concentration-dependent ARE activation via the Nrf2-Keap1 pathway [33]. Krifka et al. found that HEMA directs the differential expressions of Nrf2-regualted antioxidant enzymes, such as SOD, CAT, and GPx [20,22]. A recent study by the same group provides direct proof that Nrf2 is a major regulator of metabolic pathways activating cellular

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Table 1. Summary of phase II xenobiotic conjugation enzymes.

Enzyme	Description	Reference
Heme oxygenase-1 (HO-1)	Catalyzes first, and rate-limiting, step of heme degradation; expression induced by oxidative stress	[37]
Superoxide dismutase (SOD)	Catalyzes dismutation of the superoxide anion radical (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2)	[19]
Catalase (CAT)	Catalyzes hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2)	[38]
Glutathione peroxidase (GPx)	Catalyzes hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2)	[38]
Glutathione-S-transferase (GST)	Catalyzes the conjugation of GSH to hydrophobic electrophiles and aid in their excretion	[39]
NADH quinone oxidoreductase 1 (NQO1)	Obligate enzyme that catalyzes two-electron reduction and detoxification of redox cycling compounds including quinones	[40]
Glutamyl-cysteine ligase	Composed of a modulatory and a catalytic subunit (GCLM/GCLC). First enzyme in glutathione (GSH) biosynthesis: L-glutamate + L-cysteine + ATP ↔ gamma-glutamyl cysteine + ADP + Pi	[41]

For further information, see [27].

responses to maintain redox homeostasis in HEMA-exposed cells, and that the activation of the Nrf2-regulated antioxidant cell response by Nrf2 activator can inhibit HEMA-induced oxidative stress and support cell viability [34]. These finding add more weight to our hypothesis that activation of Nrf2 can eliminate resin-associated oxidative damage.

Testing the Hypothesis

To prove this hypothesis, several sets of experiments are required. First, to explore the relevance of EGCG to resin monomer cytotoxicity, an *in vitro* experiment is needed with human dental pulp cells obtained from a primary culture, according to a protocol approved by the Ethics Committee of the Fourth Military Medical University with written informed consent obtained from all subjects, which previously have been demonstrated to be sensitive to resin monomer cytotoxicity [35]. The grouping of this experiment is as follows: cells are treated with a range of doses of resin monomers alone (positive control cells) or in combination with EGCG. Negative control cells are left untreated. Then the cells will be cultured at a series of time points (1 h, 2 h, 6 h, 12 h, and 24 h). To study the changes of cell proliferation of the cells treated with monomers in presence or absence of EGCG, CCK-8 (cell counting-8) will be used. Dental monomers have been proved to disturb intracellular redox equilibrium and cause oxidative stress with overproduction of ROS and depletion of reduced GSH. Thus, intracellular ROS levels and GSH levels will be measured with DCFH-DA staining and GSH/GSSH assay kit, respectively. The induction of apoptosis cause by resin monomers is related to oxidative stress, so next we will perform Annexin V/propidium iodide double-staining to study the apoptotic effects of dental monomers and possible protective effects of EGCG. The hypothesis predicts that co-treatment of cells with EGCG and dental monomers can salvage cell viability and block the apoptotic effects of dental monomers completely, as well as suppress over-generation of ROS and depletion of GSH.

The next set of experiments is designed to illustrate the mechanism underlying EGCG-directed detoxification of dental monomers at the molecular level. In normal conditions, Nrf2 is kept transcriptionally inactive by bonding to Keap1. When cells are exposed to oxidative or electrophilic stress, Nrf2 dissociates from Keap1 and subsequent nucleus translocation of Nrf2 and induces the expression of its ARE-regulated target genes, including the genes that encode phase II detoxifying and antioxidant proteins. Therefore, we will perform immunofluorescence to monitor the nucleus translocation of Nrf2. To measure the transcriptional activities of ARE promoter, a luciferase assay will be performed. Then qRT-PCR (Quantitative reverse transcription polymerase chain reaction) and Western blot will be performed to measure transcription and expression of antioxidants and phase II enzymes, including HO-1, SOD, CAT, GPx, GST, and NQO1. The hypothesis predicts that nucleus immunofluorescence is stronger in the presence of EGCG and dental monomers than that with dental monomers alone. These results would confirm that Nrf2 escapes from Keap1 and Nrf2 nucleus translocation. The transcriptional activation of ARE promoter confirms after Nrf2 accumulates in the nucleus, it binds to AREs and transcriptionally activates ARE. The elevated mRNA and protein levels of ARE-regulated target genes would further confirm the activation of Nrf2 pathway and expression of antioxidants and phase II enzymes, which contributes to EGCG-induced detoxification of dental monomers cytotoxicity.

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We will perform further experiments in an *in vivo* dog pulp model to investigate the preservation of the direct capping pulp in deep caries, as described before [36]. The animal study was approved by the Ethics Committee of the Fourth Military Medical University. These individually caged dogs will have regulated light and temperature and will be fed dog chow and water. A total of 36 teeth (4th pre-molar or 1st molar) of 6 healthy male beagle dogs (18–24 months old, weighing 8–10 kg) will be used. The animals will receive anesthesia by intramuscular injection of 20 mg/kg ketamine HCl and 0.05 mg/kg acepromazine. Three groups will be tested for direct pulp capping:

1. Negative control with no pulp-capping material;
2. Positive control with Dycal radiopaque calcium hydroxide (Dentsply, Milford, DE) without additives;
3. Experimental group: Dycal with EGCG.

The additive amounts of EGCG are based on the results of *in vitro* experiments. Two time intervals of 10 days or 60 days will be used to monitor pulp response. Thus, 8 teeth will be enrolled for each group (n=8). After local anesthesia and disinfection, Class V cavities will be prepared on the buccal surface of the teeth to expose the pulp. The pulp exposures produced will be about the same size (1 mm). Following exposure of the pulp, the access cavity will be rinsed with sterile saline solution and dried with sterile cotton pellets with light pressure to control the bleeding. The pulp exposures will then be covered with the materials of each group, as described above. Then, all the cavities for all 4 groups will be filled with a resin-modified glass ionomer cement (GC Fuji, Japan). After 10 or 60 days, the dogs will be sacrificed and the teeth and surrounding tissues will be dissected from the jaws. For each tooth in the 10-d evaluation, the sections will be stained with hematoxylin and eosin (H&E) for histomorphologic analysis. The sections will be examined for pulp response and reparative dentin formation. For each tooth in the 60-day evaluation, the thickness of the reparative dentin formation will be measured for all the sections throughout the exposure field using Adobe Photoshop software CS6. The average value will be recorded as the reparative dentin thickness of the tooth. Hence, 6 thickness values for each group will be obtained for statistical analysis (mean ±SD; n=8).

The hypothesis predicts that there is more inflammatory infiltration in negative or positive controls than in the experimental group, which contributes to anti-inflammatory and antioxidant properties of EGCG. However, no dentin bridge will be noticed in any group, because the time period is not long enough. At 60 days, for the experimental group, substantial reparative dentin formation with dentin tubules will be observed, as well as numerous dentinal tubule lines and odontoblasts in it. In contrast, in negative controls and positive controls there will be no reparative dentin bridge formation and necrosis will be seen in pulps with chronic inflammation reaction persisting in pulpal tissues. These results will demonstrate that EGCG can improve the effects of pulp capping materials by inducing more reparative dentin formed without inflammatory response.

Significance of the Hypothesis

To the best of our knowledge, the relationship between EGCG and dental monomer-caused cytotoxic effects has never been put forward. We consider that EGCG may eliminate dental monomer-induced cytotoxic effects by activation of the Nrf2-Keap1 pathway. If this hypothesis is proven, it may help better define the mechanism of dental monomer-induced cytotoxicity and even help develop innovative resin materials with better biocompatibility.

Statement

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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