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THE EXAMINATION OF THE RELATIONSHIP BETWEEN BRAIN DAMAGE AND DENTAL RESTORATIVE FILLING MATERIALS: AN IN-VIVO STUDY

Beyin Hasarı ile Restoratif Materyaller Arasındaki Bağlantının İncelenmesi: Bir İn-vivo Çalışma

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ABSTRACT

The aim of this study is to investigate the possible effects of restorative materials such as resin composite, amalgam and glass ionomer on brain tissue. Thirty-two Wistar albino rats were taken and restorative materials were put in the neck region at the back. After 8 weeks, the brain tissues were removed and analyzed using nuclear factor kappa B (NF-κB)/p65 and hematoxylin & eosin staining methods. Further, the levels of superoxide dismutase, catalase, lipid peroxidase, and glutathione were determined in the brain tissues. More intense staining of immunopositive cells was observed in the restorative material groups than the control group, also neuronal degeneration was detected in these groups. Results of the tests indicated increased oxidative stress in all the restorative material groups compared with the control group. All three dental restorative materials exhibited cytotoxic effects on the brain tissue. Additionally, oxidative stress may have occurred in the brain tissue. The heavy metal compounds in the restorative materials caused neuronal degeneration and may also have caused oxidative stress in the brain tissue, indicating the cytotoxic effects of dental restorative materials.

Keywords: Brain Degeneration, Dental Amalgam, Glass Ionomer Cement, Oxidative Stress, Resin Composite

ÖZ

Bu çalışmanın amacı, rezin kompozit, amalgam ve cam iyonomer gibi restoratif materyallerin beyin dokusu üzerindeki olası etkilerini araştırmaktır. Otuziki adet Wistar albino sıçan alındı ve restoratif malzemeler boyun bölgesinin arkasına yerleştirildi. 8 hafta sonra beyin dokuları çıkarıldı ve nükleer faktör kappa B (NF-Kb)/p65 ve hematoksilin & eosin boyama metodları ile analiz yapıldı. Ayrıca, beyin dokusunda; süperoksit dismutaz, katalaz, lipid peroksidaz ve glutatyon düzeyleri tespit edildi. Deney gruplarında kontrol gruplarına göre immünpozitif hücre boyanması daha yoğun olarak gözlendi, ayrıca bu gruplarda nöronal dejenerasyon saptandı. Test sonuçları, tüm restoratif materyal gruplarında kontrol grubuna kıyasla artmış oksidatif stres gösterdi. Kullanılan restoratif materyallerin üçü de beyin dokusu üzerinde ciddi sitotoksik etkilere neden oldu. Ek olarak, beyin dokusunda oksidatif stres oluşmuş olabilir. Restoratif maddelerinin içerdikleri ağır metal bileşikler, restoratif maddelerin sitotoksik etkilerini gösterecek şekilde nöronal dejenerasyona neden oldu ve ayrıca oksidatif strese yol açmış olabilirler.

Anahtar kelimeler: Beyin Dejenerasyonu, Cam İyonomer Siman, Dental Amalgam, Oksidatif Stres, Rezin Kompozit

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INTRODUCTION

Kidney, oral, and ophthalmic diseases have the second greatest effect on public health after infectious diseases (Glick et al., 2012). Although oral diseases can be prevented and/or treated, they are among the most prevalent diseases worldwide. Additionally, there is a high prevalence of dental decay among the population (Uzer Celik & Tunac, 2016). Dental decay can easily be treated with restorative materials. However; these restorative materials can affect the oral flora and potentially cause adverse reactions (Tillberg, Jarvholm, & Berglund, 2008).

Conferences related to oral health have been annually arranged by the World Dental Federation (FDI), the International Association for Dental Research (IADR) and the International Dental Manufacturers (IDM) since 2009. The hot topics at these conferences are about dental restorative material types, properties, usages, and alternatives (Petersen, Baez, Kwan, & Ogawa, 2009). Amalgam, resin composite, and glass ionomer are the popular restorative materials used to prevent dental decay (Tillberg et al., 2008). The use of resin composites and glass ionomers for dental restorative care is suggested instead of using amalgam (Petersen et al., 2009).

According to the literature, oxidative stress plays a significant role in the development of severe illnesses, including neurodegenerative disorders such as lateral amyotrophic sclerosis, Parkinson's disease, and Alzheimer's disease (Guest & Grant, 2012).

Restorative materials are polymers composed of different monomers. Sometimes these monomers can easily pass through the pulp, which contains the neurovascular bundle, into the bloodstream. Mercury (Hg) is the most significant chemical element in amalgam. Mercury is thought to play a possible role in the development of Parkinson's disease, Alzheimer's and multiple sclerosis (Needleman, 2006). Glass ionomer cements are calcium-based structures dependent on the reaction of acid (a water-soluble polymer)/base (strontium aluminosilicate glass powder). The material has been developed in dentistry as a dental restorative material that releases fluoride over a long period, bonds to the surface of the tooth and it is highly biocompatible (Donly & Henson, 2005). Additionally, aluminum is the primary ingredient of ionomer glass cements. The aluminum is released from the glass into the polyalkeonic acid solution through mixing and setting. When the glass ionomer cement is polymerized, the amount of aluminum released reduces as the aluminum ions near the surface are washed away from the cement and the remaining ones are trapped deep inside the matrix (Nakajima, Komatsu, & Okabe, 1997). On the other hand, the various commercial resin-based restorative

materials have different chemical, biological, physical, and clinical features. Most scientific studies have shown differences with respect to hardness, compressive strength, tensile strength, water sorption, conversion of the methacrylate groups, color stability, abrasion, *in vitro* cytotoxicity, and antibacterial activity (Lammeier et al., 2012). All the components and compositions of each dental material are shown in Table 2.

Dentists desire the development of new alternative dental restorative materials. Alternative dental restorative materials are recommended in terms of health safety, dental field advancement, long-term efficiency, potential adverse effects and material viability. It is necessary to obtain more detailed information about the side effects of dental restorative materials. Long-term monitoring is also necessary. Consensus on restorative procedures showed the need for further study of approaches to the use of amalgam (Petersen et al., 2009).

The aim of this study was to evaluate the possible effects of dental restorative products, including resin composite, amalgam and glass ionomer on rat brain tissue. We used parameter tests of histopathological, immunohistochemical, and oxidative stresses.

METHODS

Thirty-two female Wistar albino rats (250–300 g) were purchased from the Experimental Animal Laboratory (Approval No: 42190979-01-02-339-93). The rats, as described below, were divided into four groups (Table 1).

Table 1. Experimental Design of All Groups

	Group's Name	**N**
Group 1	Control group (Negative Control)	n=8
Group 2	Dental amalgam group (20 mg/kg)	n=8
Group 3	Glass ionomer group (20 mg/kg)	n=8
Group 4	Resin composite group (20 mg/kg)	n=8

In this study, specimens were prepared using the three most frequently used types of dental restorative materials. All specimens were prepared in the form of small spheres of 20 mg Thiopental sodium (pentothal sodium) (Abbott Lab, Istanbul, Turkey). The dental restorative materials used were nano hybrid resin composite, non-gama-2 capsule dental amalgam, and glass ionomer cement (Table 2).

Table 2. The Components and Compositions of Each Dental Material

Classification	Composition
Dental amalgam	• Silver 45% • Copper 24% • Tin 30% • Zinc 0,5% • Mercury
Glass Ionomer Cement	• Fluoro Aluminosilicate glass 90% • Polyacrylic acid 10% **Typical percentage of glass ionomer cement powder:** • Silica 41.9% • Alumina 28.6% • Calcium Fluoride 15.7% • Sodium Fluoride 9.3% • Aluminum Phosphate 3.8% • Aluminum Fluoride 1.6%
Nano hybrid Composite	• Bisphenol A-glycidyl methacrylate (Bis-GMA) • Triethylene glycol dimethacrylate (TEGMA) • Urethane dimethacrylate (UDMA) • Zirconia/silica fillers • Polyethylene glycol dimethacrylate (PEGDMA) • 2,2 bis(4-(2-methacryloxyethoxy) phenyl) propane (Bis-EMA)

Nano hybrid resin composite specimens were cured with a LED light source (Valo LED, Ultradent, South Jordan, ABD) for 40 s. Capsule dental amalgams were mixed with amalgamator (RotoMix, 3M ESPE, Schaumburg, USA). Mixed amalgams were separated into small 20 mg spheres, and then the specimens were maintained for a 48-h setting time. Glass ionomer cement (GIS) capsules were mixed with an amalgamator. Mixed GIS were separated to 20 mg small spheres, and then the specimens were maintained for a 24-h setting time.

All restorative materials were subjected to ethylene oxide sterilization to avoid any bacteremia. After sterilization, specimens were placed in special sterile sealed bags for 1 week, and the degassing method has been introduced.

Both surgical procedures using thiopental sodium as an anesthetic have been conducted under sterile conditions. Throughout the acclimatization era, rats were fed with a diet of normal commercial rat pellets. Animals were anesthetized with thiopental sodium 25 mg / kg for this procedure and implanted intraperitoneally. Restorative materials were subcutaneously applied to the back of the neck region in all experimental groups. Rats were maintained for 8 weeks before sacrifice. In the negative control group (Group 1), the rats were not treated with any restorative materials. The rats were sacrificed at the end of the study in compliance with the ethics guidelines.

Samples of rat brain tissues were obtained and preserved at -80 ° C for 3 days. Tissues were covered with liquid nitrogen to create homogeneous sample. The tissues (0.5 g) were

combined with the solution (4.5 mL) and the samples were placed in a homogenizer (IKA T18 Ultra-Turrax, Merck KGaA, Darmstadt, Germany) and homogenized on ice for 15 minutes. The samples were processed and centrifuged at 15000 rpm for 15 min and the supernatants were used for study. All analyzes were conducted at room temperature in triplicate.

After removal, rat brains were quickly set at 10% formalin for 72 h for histopathological and immunohistochemistry experiments. Samples have been dehydrated in a standardized alcohol chain, submerged in xylene, and sealed in paraffin wax. Then, 5 µm sections were obtained with a microtome (Leica RM2125RT, Leica Biosystems Nussloch GmbH, Nussloch, Germany) from a paraffin block. Sections have been colored with hematoxylin & eosin for examination in light microscope (Nikon Eclipse E600, Nikon Instech Co., Kanagawa, Japan) and photographed with a light microscope with a microscope camera system (Olympus DP72, Olympus Optical Co., Tokyo, Japan).

In addition to histopathological analyses, the nuclear factor kappa B (NF-κB)/p65 marker was used for determination of oxidative stress on the cellular level. Staining of the NF-κB protein was conducted using an automated baking-through-staining solution (BenchMark GX System, Roche Diagnostics, Arizona, USA). The main antibody of NF-κB/p65 (NF-κB p65 Antibody (F-6) Cat No: sc8008, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) was used at a dilution of 1:80 for 30 min at 37°C. The specimens were first incubated with the diluted antibody used as a chromogenic agent, followed by a universal detection kit (UltraView Universal DAB Cat No: 05269806, Roche Diagnostics, Arizona, USA). Hematoxylin (Hematoxlin Cat No: 05266726, Roche Diagnostics, Arizona, USA) was used as a counterstaining agent.

In order to determine the significance of the observed differences, we used a one-way ANOVA with Duncan test. All statistical analyses have been made using software (SPSS 15.0 for Win, IBM Software, New York, USA). Statistical significance was determined at $p<0.05$.

RESULTS

Lipid peroxidase (LPO) is a biological marker for tissue damage. When LPO levels were measured in our study, there were significant differences ($p<0.05$) between the negative control and other groups (dental amalgam, resin composite, and glass-ionomer). The largest increase ($p<0.05$) in LPO level was detected in Group 3 (glass-ionomer group). On the other hand, there were also significant differences ($p<0.05$) between Group 2 and 3 (dental

amalgam and glass-ionomer), and Groups 2 and 4 (dental amalgam and resin composite) (Table 3).

Table 3. Biochemical Results of Brain Tissues (Mean±Standard Deviation)

Groups	**LPO Level (nmol MDA/g tissue)**	**SOD Activity (mmol/min/mg tissue)**	**CAT Activity (µmol/min/mg tissue)**	**GSH Level (mmol/mg tissue)**
Control	14,667±0,27[a]	0,096±0,02[a]	0,076±0,04[a]	0,742±0,08[a]
Dental Amalgam	16,845±0,11[b]	0,274±0,07[b]	0,106±0,03[b]	0,448±0,08[b]
Glass ionomer	21,289±0,4 [c]	0,062±0,02[a]	0,1±0,04[b]	0,755±0,07[a]
Resin composite	20,178±0,25[b,c]	0,241±0,04[c]	0,086±0,01[a,b]	0,533±0,06[c]

* Superscripts in each column show statistical differences.

Brain superoxide dismutase (SOD) activity was significantly different between all groups ($p<0.05$). Dental amalgam increased SOD activity by the control and resin composite groups. However, a significant decrease in SOD activity was observed for the glass ionomer group. Additionally, there were significantly differences ($p<0.05$) among the experimental groups, when compared with each other (Table 3).

The catalase (CAT) activities of all groups were measured, and it was shown that there were significant differences between the dental amalgam, glass-ionomer and control groups ($p<0.05$). Furthermore, there was no significant difference between the control and resin composite groups ($p>0.05$). The CAT activity levels of the control and resin composite groups were nearly at the same level (Table 3).

Finally, glutathione (GSH) levels were detected in all experimental groups. The highest GSH level was observed in the glass-ionomer group, but no differences were detected when compared with the control group ($p>0.05$). Alternately, the lowest GSH level was detected in the dental amalgam group. A dramatic decrease in GSH level was detected in the dental amalgam and resin composite group brain tissues, as shown in Table 3.

In our study, conventional light microscopic examination was performed using both hematoxylin & eosin and NF-κB staining methods. When hematoxylin & eosin staining of control group tissues was evaluated, all brain tissue appeared normal including the blood vessels, neurons, and glial cells (Figure 1A). Hyalinization in the brain section was remarkable in the resin composite group. Additionally, there were some degenerative cells with hyperchromatic cells in the resin composite group (Figure 1B). Neurons belonging to the glass ionomer and dental amalgam groups showed more shrunken and hyperchromatic nuclei, as shown in Figure 1C and 1D.

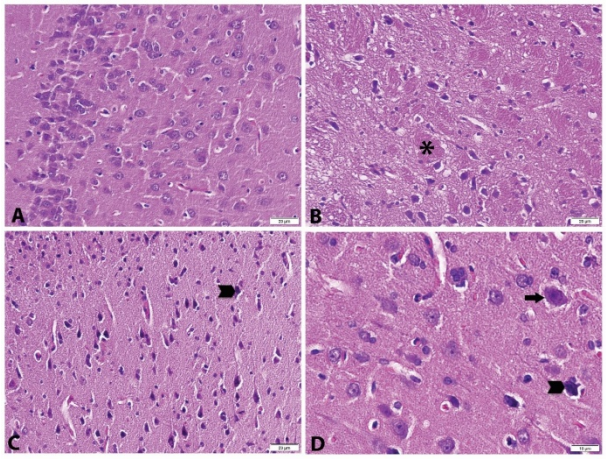

Figure 1. Light microscopic photomicrograph of all groups. **(**Hematoxylen&eosin)
A: Control group (the brain tissue was normal appearance), **B:** Resin composite group (Hyalinization were seen in some areas), **C:** Glass ionomer group (Hyperchromatic neurons were detected), **D:** Dental amalgam group (Hyperchromatic and degenerative neurons),➧: neuron with hyperchromatic nucleus, ➡**:** degenerative neuron, ***:** hyalinized area in brain tissue.

Immunohistochemical results

For the immunohistochemical examination, NF-κB p65 staining was performed on tissues of all experimental groups. No NF-κB-positive cells were detected in the control groups (Figure 2A), whereas many NF-κB positive cells were detected in the resin composite, glass ionomer, and dental amalgam groups (Figure 2B-D).

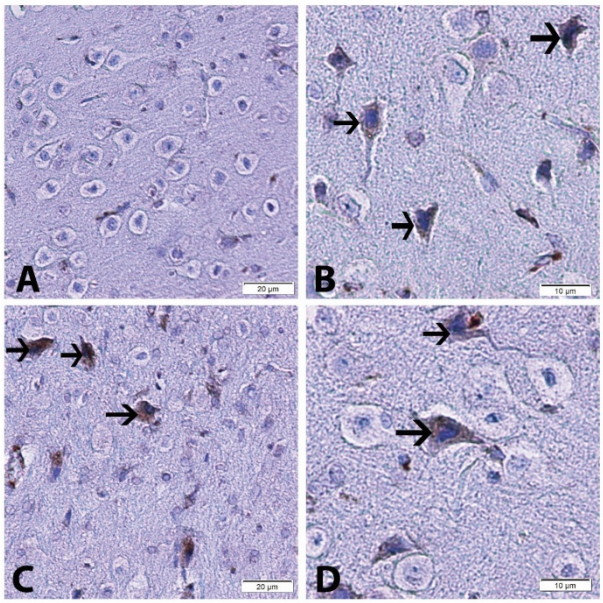

Figure 2. Light microscopic photomicrograph of all groups. (NF-κB p65)
A: Control group (There were no immunpositive neurons), **B:** Resin composite group, **C:** Glass ionomer group, **D:** Dental amalgam group, (Immunpositive cells were detected in resin composite, glass ionomer and dental amalgam groups)**,**➡**:** NF-κB immune positive cells.

DISCUSSION

Dental amalgam is an alloy composed of mercury, silver and other chemicals of different quantities. Amalgam may also contain other heavy metals such as platinum, cadmium, and palladium. Many in vivo and in vitro experiments have established that amalgam is the primary cause of mercury exposure and, is one of the dental restorative materials that is cytotoxic to human body tissue (Al-Khafaji et al., 2020). It is well-known that mercury is a heavy reactive metal that is toxic at high doses, especially in some areas of the brain (Ratcliffe, Swanson, & Fischer, 1996). Mercury exposure has also been shown to be a risk factor for multiple sclerosis and Alzheimer's disease (Thompson, Markesbery, Ehmann, Mao, & Vance, 1988).

Colloidal mercury can oxidize and accumulate in the brain by crossing the blood-brain barrier. The brain damaging mechanism may be explained by the biological half-life of Hg in the brain. The half-life of Hg in the brain is not fully clear, but is calculated to be as long as 20 years. Colloidal mercury is closely bound to selenium or SH-groups after brain oxidation, which can lead to the brain's residual deposits and cause brain damage (Park & Zheng, 2012). Our results regarding dental amalgam showed that the brain was dramatically affected by long-term mercury exposure. The histological and immunohistochemical data showed that neurons exposed to dental amalgam were shrunken, exhibited hyperchromatic nuclei, and contained many immunopositive cells.

Rossi et al. (Rossi et al., 1997) showed that Hg can induce apoptosis in cerebellar granule cells by affecting calcium channels. This induces a distribution of antioxidant mechanisms and an increase the types of reactive oxygen that cause DNA damage. It is known that amalgam drastically increases oxidative stress (Pizzichini et al., 2002). Mercury levels were observed to be normal in the control group, whereas amalgam restorations lead to increased oxidative stress and reduced GSH levels, thus inducing cellular damage. In addition, CAT, SOD, and LPO levels were significantly increased in amalgam-exposed brain tissues compared to the control group. This indicates that brain damage may lead to oxidative stress-mediated enzyme activity increases. The cells were damaged during oxidative stress by the disruption of DNA and some proteins.

Resin composites are composed of non-polymerized monomers. The commonly used monomers of resin composites are triethyleneglycoldimethacrylate (TEGDMA), hydroxyethylene methacrylate (HEMA), bisphenol-A-glycidylmethacrylate (BisGMA) and urethanedimethacrylate (UDMA). TEGDMA and HEMA molecules bind to the cell

membrane lipid structure, affect membrane permeability, and have been shown to cause cell death (Miyatake, Miyagawa, Mizuo, Narita, & Suzuki, 2006). According to the immunohistochemical results, there was more intense staining of immunopositive cells in the resin group compared with the control group. Additionally, degenerative cells, hyperchromatic cells, and hyalinization in brain sections were observed in the histological sections from the resin group. The cytotoxic effects of resin composites on some human tissues have been shown (Aranha, Giro, Hebling, Lessa, & Costa, 2010). The biochemical antioxidant parameters also showed oxidative damage to brain tissue. The levels of antioxidant enzyme activities were similar to those of the dental amalgam group. SOD, CAT and LPO levels increased while GSH levels decreased in resin composite-exposed animals. GSH inhibits tissue damage by maintaining lower levels of reactive oxygen species (ROS) at determined concentrations (Polat et al., 2011). In this research, we noticed that resin composite application decreased GSH levels relative to the control group. It is well-known that lipid peroxidation increases under inflammatory conditions (Uzkeser et al., 2012). As a marker of inflammation and oxidative damage, lipid peroxidation shows changes in membrane permeability. Therefore, LPO may lead to an increase in the amount of protein degradation, which eventually results in cell lysis (Garcia et al., 1997).

Glass ionomer cements are used as bonding for crowns and as temporary and permanent restorative materials. The most important feature of these materials is the high amount of fluoride released. Glass ionomer cements also contain high amounts of aluminum ions and are water-soluble (Christensen, 1990). In one study performed by Crapper et al., it was shown that the aluminum ions of glass ionomers caused neuronal damage in the brain tissue and may affect the learning center of the brain (Crapper, Quittkat, Krishnan, Dalton, & De Boni, 1980). Campbell et al. reported that the aluminum ion caused neuroblastomas and gliomas (A. Campbell, Kumar, La Rosa, Prasad, & Bondy, 2000). Brain damage was also demonstrated in the glass ionomer group. Shrunken and hyperchromatic nuclei as well as many immunopositive cells were detected by histopathological and immunohistochemical examination. Additionally, some notable changes in oxidative stress parameters (CAT, SOD, GSH, and LPO) were detected in the glass ionomer group.

Oxidative stress-mediated tissue damage may be reversed by SOD and GSH. The activity of these two antioxidant factors should inhibit the cytotoxic impact of toxic free radicals. (Omar, Flores, & McCord, 1992). According to our results, the levels of oxidant and antioxidant enzymes were not in normal ranges when compared to the literature. (Tong. et al.,

2018; Uikey, Hazarey, & Vaidhya, 2003) Especially, SOD levels were quite decreased in experimental groups. However, in our study the levels of enzyme parameters were compared with the mean values of control groups levels instead of reference ranges due to the differences between blood and tissue enzyme levels. SOD plays a key role in preventing damage by turning the superoxide into the less reactive hydrogen peroxide that can be broken down by CAT. In our study, we observed decreased SOD activity. The decrease in SOD activity caused an increase in CAT activity, as shown in Tables 4 and 3. The increase in CAT activity suggests a rise in the amount of hydrogen peroxide. CAT is a highly reactive enzyme that interacts with hydrogen peroxide to generate water and molecular oxygen. (Polat et al., 2011). Kamendulis et al. detected increased activation of catalase activity in neurons and glial cells (Kamendulis, Jiang, Xu, & Klaunig, 1999). However, Gills et al. did not observe any significant difference in the damage caused by aluminum to other organs (Campbell, Prasad, & Bondy, 1999). Finally, all results showed that dental restorative materials had cytotoxic effects on brain tissue in different rates. However, if it is necessary to point out the less and more toxic materials, they were resin composite and glass ionomer, respectively.

Our results showed that amalgam, glass ionomer cement, and resin composite had serious cytotoxic effects on brain tissue through a range of mechanisms that are still unclear. Heavy metal derivatives of these restorative products have degenerated the neurons. In addition, oxidative stress may also exist in brain tissue, contributing to the cytotoxic effects of dental restorative materials.

In our study, restorative materials were placed in the back of rats. This may have facilitated the passage of metal compounds and ions into the blood. Similar studies can be done in larger experimental animals by placing restorative materials in their dental cavities.

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