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Original research

Release of mercury from amalgam filling and its relationship with metallothionein and superoxide dismutase

Purpose
This study aims at determining the amount of mercury released over time from amalgam after treatment in healthy subjects and to examine the relation of mercury with serum MT-1 and SOD-1 levels.

Materials and Methods
Amalgam filling was applied to the 15 subjects aged 19-22 years and blood samples were collected before treatment and 1 day, 7 days, 21 days and 35 days after treatment. Mercury analysis of serum samples was performed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). In addition, MT-1 and SOD-1 levels in serum samples were measured using commercial enzyme-linked immunosorbent assay (ELISA). Friedman test and Spearman's correlation analysis was performed to analyse the data. p value was interpreted in significance level of 0.05.

Results
As a result of the analysis for MT-1, it was found that the values decreased over time and this decrease was statistically significant after 21 days ($p<0.05$). In addition, it was found that SOD-1 decreased over time, but this decrease was not statistically significant . In terms of released mercury, there was no statistically significant difference among the values of mercury released over time . According to the results of correlation analysis, no statistically significant relationship was found among the variables.

Conclusion
The results of the present study indicated that the amount of mercury released from the tested amalgam were found to be tolerable and no significant relationship was found between MT-1 and SOD-1.

Keywords: *Amalgam, antioxidants, mercury, metallothionein, superoxide dismutase*

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Introduction

Dental amalgam is a restorative material that has been used in dentistry for many years. The main ingredients of amalgam alloy are silver (Ag), tin (Sn), copper (Cu) and mercury (Hg), but it also contains small amounts of zinc (Zn) and palladium. Although amalgam is an easy to handle, durable and cheap material, its use has become questionable due to its inability to meet aesthetic criteria, low dimensional stability and also Hg in its content (1). Since amalgam fillings are exposed to chemical, biological, mechanical and thermal effects in the mouth, they are in constant interaction with the oral environment and the most important part of this interaction is the corrosion of amalgam (2). Also, excessive chewing of gum increases the wear of amalgam fillings and the Hg release rate (3).

Metallothionein (MT) is a low molecular weight protein rich in intracellular cysteine and has the capacity to bind heavy metal ions such as cadmium (Cd), Zn, Hg and Cu. MT binds metal ions via the thiol (-SH) group of cysteine resi-

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dues. MT shows a high affinity for both basic metals such as Cu and Zn and non-essential (or toxic) metals such as Hg and Cd (4). MTs found in humans are four classes (MT1-MT4) with multiple isoforms of MT-1 and MT-2, and MT-1 and MT-2, in particular, play a very crucial role in the distribution and storage of elements such as Hg in the body (5). Metallothioneins have many crucial functions in the regulation of Zn and Cu homeostasis, removal of heavy metals (6). Cell proliferation and differentiation and scavenging of free radicals (4,7). Previous study reported that MT can act as a free radical scavenger and metallothionein scavenges hydroxyl radicals in vitro (8).

Thornalley and Vasak (8), reported in their study that rabbit liver MT-1 can scavenge free hydroxyl (•OH) and superoxide (O2−•) radicals produced by xanthine/xanthine oxidase. Superoxide Dismutase (SOD) is an antioxidant enzyme with metal itons in its active center. There are three types of SOD, two of which are cytoplasmic (Cu/Zn SOD) bound to Zn and Cu, and the other extracellular SOD (Ec SOD) (9).

Humans are exposed to Hg released from amalgam as a result of swallowed saliva and direct absorption to blood via oral mucosa (1). It has been reported in the literature that Hg forms reactive oxygen species (ROS) by interacting with protein-bound -SH groups and can cause oxidative damage in tissues by affecting mechanisms such as lipid peroxidation (10). It has been reported that cells can remove ROS by producing proteins such as glutathione and metallothione that have the ability to bind to ROS (11). In addition, Cu and Zn including superoxide dismutase (Cu/Zn-cytoplasmic enzyme (SOD-1) protects the cell against ROS toxicity by metabolizing superoxide radicals to molecular oxygen and hydrogen peroxide (12).

This study aims at determining the amount of Hg released over time from amalgam after treatment in healthy subjects and to investigate the relationship of Hg with serum MT-1 and SOD-1 levels. The main null hypothesis of the study is that Hg released from amalgam do not affect serum MT-1 and SOD-1 levels of subjects.

Material and Methods

Ethical statement

This study was approved by Atatürk University Faculty of Dentistry Ethics Committee (20.11.2014/031) and was conducted in accordance with the Declaration of Helsinki. All subjects included in the study were informed about the study and written consent was obtained from the subjects that they were informed about the study. The study was conducted with 15 subjects aged between 19-22 (21.11±1.32).

Sample size determination

The sample size was determined by G*Power 3.1.9.4 software (Heinrich-Heine Dusseldorf University, Dusseldorf, Germany) using the following parameters: 95% power, 0.38 effect size, and α error at 0.05. It was determined that the appropriate sample size should be a minimum of 15 persons.

Study design

Subjects who did not have systemic disease, did not take any medication at least 3 months ago, did not smoke or consume alcohol, did not have bruxism or chewing gum habit, did not have periodontal disease and did not have fillings in their mouths were included in this study.

The amalgam filling material (Cavex Avalloy, Cavex Co., Holland, lot no:130523) was applied to the subjects according to the manufacturer's instructions. According to the information given by the manufacturer, the content of amalgam used in the present study was Cu 24.0%, Zn 0.5%, Sn 30.5%, Ag 45.0%. The mixing ratio is: 10 parts of alloy to 10.3 parts of Hg. Subjects were asked not to eat on the day of the test and fillings were made between 9-12 in the morning. Maximum 3 occlusal restorations were applied to subjects. One day after the treatment, the filling surfaces were polished. Rubber dam was used during polishing. The masses of the amalgam fillings were weighed and recorded in milligrams. (10, 13). Blood samples were collected baseline and 1 day, 7 days, 21 days and 35 days after treatment. Blood samples were obtained by collecting venous blood into test tubes as in routine procedure. After centrifugation (1000 rpm/20 min), the serum samples were kept at –80 °C until analysis.

MT-1 and SOD-1 analysis

MT-1 levels in serum samples were measured using commercial enzyme-linked immunosorbent assay (ELISA) (human MT-1, Cloud-Clone Corp., Houston, TX, USA, Lot No: L150626819), and SOD-1 levels were determined by ELISA kits (human SOD-1, Cloud-Clone Corp., Houston, TX, USA, Lot No: L160203202) according to the manufacturer's recommendations.

Testing protocols

MT-1 and SOD-1 analysis was performed by a similar method. These kits include a microtiter plate which is pre-coated with an antibody specific for MT-1 or SOD-1. Then, standards or samples is added to the wells of the suitable microtiter plate with MT-1 or SOD-1 specific biotin-conjugated antibody. Later, Horseradish Peroxidase conjugated Avidin is incubated after adding it to the entire microplate well. TMB substrate solution is then added. After this procedure, only wells containing MT-1 or SOD-1, biotin-conjugated antibody, and enzyme-conjugated Avidin will show a change in color. By adding sulfuric acid solution, the enzyme-substrate reaction is terminated and a color change occurs. This change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm.

The concentration of MT-1 or SOD-1 in the samples is determined by comparing the Optical Density of the samples to the standard curve. For MT-1, the standard curve concentrations used for the ELISA's were 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL. Detection range of MT-1 is 31.2-2,000 pg/mL. For SOD-1, the standard curve concentrations used for the ELISA's were 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL. Detection range of SOD1 is 62.5-4,000 pg/mL.

Analysis process

Assay procedure was firstly started by preparing all reagents, samples and standards. Wells for sample, diluted

standard and blank were determined. 100 µL standard or sample to each of wells was added and incubated 2 hours at 37°C. Later, aspirated and added 100 µL prepared Detection Reagent A and incubated 1 hour at 37 °C. In addition, aspirated and washed 3 times and 100 µL prepared Detection Reagent B was added and incubated 30 minutes at 37 °C. Then, aspirated and washed 5 times and 90 µL Substrate Solution was added, incubated 15-25 minutes at 37 °C and 50 µL Stop Solution was added. Then, run the microplate reader and conduct measurement at 450 nm immediately.

Sample preparation for mercury analysis

0.5 mL of serum samples were taken and transferred into teflon containers. 9 mL of HNO_3 and 1 mL of H_2O_2 solution were added on them, and the vessels and segments that make up the system were closed and placed in the microwave. Digestion of the samples was subsequently performed using an Milestone connect ETHOS UP microwave digestion system (Milestone, Sorisole, Italy) which employ a microwave program reaching 190 °C within 15 min and then held at 190°C for 15 min. 5 mL of ultrapure water was added to 10 ml samples as a result of decomposition and diluted. The samples were filtered using a 0.45 µm syringe filter and read by giving to the device without a second dilution. Each reading was the average of 3 parallel readings.

Analysis process

Hg analysis of serum samples was performed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), (Agilent 7800 series, Agilent Technologies, Japan). The ICP-MS device contains a glass MikroMist nebulizer (U-series, Australia) and a quartz spray chamber (double pass, USA) used to load samples into the system. The plasma part consists of an inert sample entry kit containing quartz torch (2.5 mm, Japan) and sample cone and skinner cone (for x-lens, USA) parts consisting of nickel material.

Before starting the analysis, the device was purged with helium gas for 45 minutes. The device was activated after parameters such as Plasma gas: 15 L/min, auxiliary gas: 1 L/min, carrier gas: 1 L/min, makeup/dilution gas: 1 L/min and carrier gas pressure 1.45 kPa were adjusted. After the device was activated, torch axis, resolution axis, EM, standard lenses tune, plasma corection, full spectrum and performance report tests were performed, respectively. Then, the calibration process of the device was carried out with tuning solution (1µg / L Ce, Co, Li, Mg, Tl, Y). The values obtained as a result of the tune operation were checked and it was determined whether there was any deviation in the device. Standard solutions prepared by using stock solutions were read and calibration curves were checked (standard reference range, for Hg element: 0, 2.5, 5, 7.5, 10 ppb). After checking the calibration curves, the samples were loaded into the device by the autosampler and analyzed. The autosampler and tubing was washed with 2% HNO_3 and ultrapure water, and the probe part was washed with a 1% HCl solution and made ready for the next injection. Measurements were made at 1200 W RF power, 1 L/min carrier gas flow and 0.30 rps nebulizer pump speed. Argon gas was used as carrier gas. Limit of detection (LOD) for Hg was 0,078 ppb and limit of quantification (LOQ) for Hg was 0.259 ppb.

Statistical analysis

Data were analysed using SPSS 20 (IBM, Chicago, IL, USA) statistical package software. Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to determine the distribution of the data. Friedman test was used to compare the levels of Hg released from amalgam fillings over time and also to compare changes in serum MT-1 and SOD-1 levels over these time periods. The relationship among MT-1, SOD-1 and Hg was examined with correlation analysis (Spearman's Correlation Analysis). In addition, Spearman's Correlation Analysis was used to determine between amalgam filling masses and released Hg. p value was interpreted in significance level of 0.05.

Results

Serum MT-1, SOD-1 and Hg values obtained from subjects and statistical analysis results are shown in Figure 1 and 2. As a result of the analysis for MT-1, it was found that the values decreased over time and this decrease was statistically significant after 21 days (180.35±64.74 pg/mL) according to baseline (349.87±36.34 pg/mL) ($p<0.05$) (Figure 1).

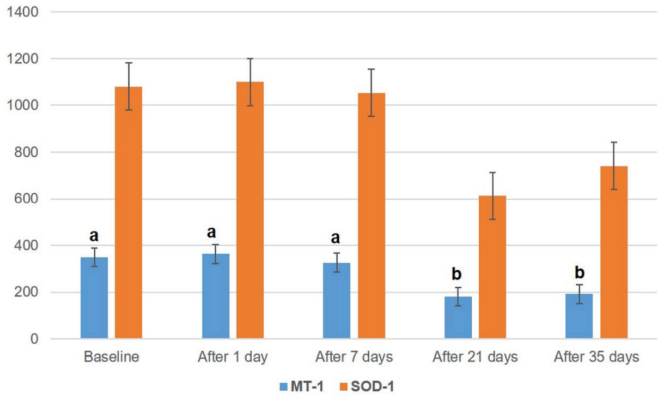

Figure 1. *Mean and standard deviation for values (pg/mL) of serum MT-1 and SOD-1 and statistical analysis results (Different lowercase letters indicate a significant difference).*

In addition, it was found that SOD-1 decreased over time, but this decrease was not statistically significant (Figure 1). According to the analysis results in terms of released Hg, no statistically significant difference was determined among the values of Hg released over time (Figure 2). Although the

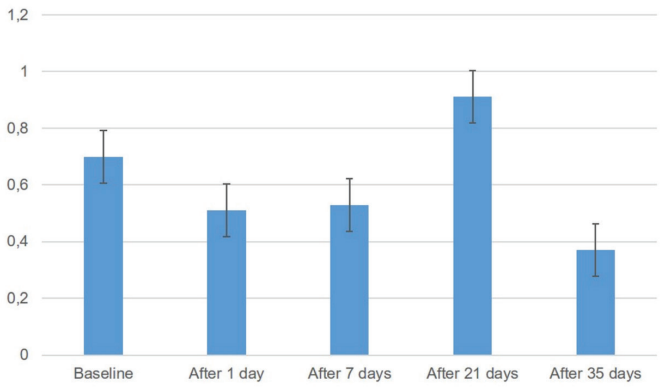

Figure 2. *Mean and standard deviation for values (ng/mL) of serum Hg and statistical analysis results.*

Hg values were not statistically significant, it was found to increase a little after 21 days.

According to the results of correlation analysis performed to determine the relationship between Hg released from amalgam and MT-1 and SOD-1 levels, no statistically significant relationship was found between the variables . Amalgam masses used in this study were found as 0.09 ± 0.08 g. No correlation was found between amalgam filling masses and released Hg .

Discussion

It has been known since ancient times that Hg in composition of amalgam, enters the body in ionic or elemental form. Exposure to the patient or dentist may occur in cases of wear or abrasion from mouth fillings (ionic form), direct evaporation of metallic mercury contained in amalgam, preparation, placement and removal of fillings (elemental mercury) (14). It is generally accepted that the short-term sudden rise in Hg concentrations during the placement, polishing or removal of amalgam fillings does not pose a significant health problem or life-threatening risk to patients (15).

The surface area of the amalgam filling and the time after treatment are the two most important variables that affect Hg release from amalgam fillings (16). Mackert and Berglund (3) stated that the Hg release rate from amalgam was on average 0.4 µg per day per amalgam surface. Al-Salehi *et al.* (16) determined that that approximately 1.0 µg/cm2 of Hg was released from four bleached amalgam surfaces within 24 hours. In this case, assuming a surface area of 1 cm^2 is equivalent to four Hg amalgam surfaces *in vivo*, it means that only 1.0 µg of Hg will be released per day into the oral cavity.

It has been reported that blood Hg levels can be used as an index to determine recent Hg^0 exposure, especially in situations of acute accidental or occupational exposure (18). In addition, measuring Hg levels in whole blood and urine is reported to be a reliable method for detecting inorganic and elemental mercury exposure (Hg) (12, 18).

It has been reported that the tolerable maximum level of Hg in the blood is 3 ng/mL (19). Skoner et al. (20) reported toxic mercury doses in the blood as 200 ng/mL and lethal mercury doses as 600 ng/mL. The results of the studies on blood Hg concentrations show differences. Melchart et al. (21) reported the amount of inorganic Hg in erythrocyte and plasma as 0.37 ng/mL and 0.38 ng/mL, respectively, and the total plasma Hg level as 0.49 ng/mL. Özdabak et al. (22) stated that the Hg measured in plasma was mainly due to amalgam fillings. Kronce et al. (23) and Ott et al. (24) reported in their study that there was no significant difference between the blood Hg concentrations of individuals with amalgam fillings and those without fillings. On the contrary, Abraham *et al.* (25) reported that the blood Hg levels of individuals with amalgam fillings were higher than those without amalgam fillings. Yildiz *et al.* (10) reported the plasma Hg levels of subjects as 5.21 ng/mL measured 24 hours after treatment. The different results from the studies might be attributed to the use of different methods to assess Hg concentrations as well as the effect of other sources of Hg, such as exposure to mercury from diet, inhaled air and drinking water. Since Hg is not taken into the body in a single way, it is difficult to blame amalgam fillings directly for the increase in Hg in the blood (19). In this respect, subjects who did not have fillings in their mouths were included in the present study in order to prevent extra Hg release that may arise from old fillings.

Hg is found in the blood like other body fluids, but its levels in the blood are quite low compared to others. Even in industrial workers exposed to high levels of Hg, measured Hg concentrations have been reported to be in the parts per billion (ppb) range (17). In the present study, serum Hg concentrations reached the highest level 21 days after treatment (0.91 ng/mL), but no statistically significant difference was found among time periods ($p=0.072$). It was found that the amounts of Hg released were low and below the maximum medically acceptable level. In present study, a maximum of three single-surface fillings (occlusal) were applied to each individual on average, and the low amount of Hg released may also be due to this situation. In order to eliminate Hg release from old fillings, participants without old fillings were included in the study. In the present study, no significant correlation was found between the released Hg and the filler masses. These results are consistent with previous studies (10, 26).

There are many studies in the literature investigating the effects of Hg on antioxidant activity systems in various tissues and fluids such as saliva and plasma (26, 27). While some of these studies found a significant relationship between Hg and antioxidants, some found no significant relationship (17, 26). Because Hg can produce reactive oxygen species, it can affect antioxidant enzymes such as SOD-1 and catalase (28). Actually, Hg produces ROS (12, 29). In this respect, enhanced SOD-1 activity helps maintain the oxidative balance in the organism, otherwise this balance could be disturbed by Hg released by dental fillings (28).

A number of mechanisms can be explained by the protective effect of MTs against Hg cytotoxicity. The most important of these is that Hg has a strong affinity for MTs due to its high -SH contents. In this respect, MT has been proposed as a biomarker of both environmental and biological monitoring reflecting metal exposure, due to its high metal binding capacity (30). However, a study examining the relationship between MT and Hg released from amalgam in human serum was no found in literature.

Metallothionein plays an effective role in controlling the intracellular metabolism of Zn and can serve as a supplier of Zn when needed (4, 6). In addition, MT has an important role in regulating the activity of Cu/Zn SOD. It is known that both MT and Cu/Zn SOD play an important role in the removal of free radicals that occur in the extracellular and intracellular environment (9). Bizon *et al.*(9) investigated the Cu/Zn SOD activity and MT concentration in the blood of non-smokers and smokers and found a negative relationship between these two variables. According to the authors, the reduction in Cu/Zn SOD activity may be due to the inactivation of the reduction reaction of $O_2\bullet\text{--}\text{--}H_2O_2$ (31). Depending on aging, an increase in MT concentration and a reduction in Cu/Zn SOD occur. This makes us think that these antioxidants may be complementary to each other.

In the present study, the MT-1 and SOD-1 levels measured in the serum of the subjects decreased after 21 days, but this decrease was found to be statistically significant for MT-1 ($p<0.05$). In addition, as a result of the correlation analysis, no significant relationship was found between the Hg released

from amalgam and MT-1 and SOD-1 levels, as well as between MT-1 and SOD-1 . As a result, the null hypothesis of the study was accepted. According to the findings of the study, we think that the antioxidant system may not have been activated due to the low amount of Hg released from amalgam.

The limitation of this study is that it was conducted with a limited number of subjects. Studies that can be carried out with more subjects will provide clearer information on the subject.

Conclusion

According to the results of the present study, the amount of Hg released from the tested amalgam were found to be tolerable and no significant relationship was found between MT-1 and SOD-1. However, more studies are needed on this subject.

Türkçe özet: *Amalgam dolgudan civa salımı ve civanın metallotionein ve süperoksit dismutaz ile ilişkisi. Amaç: Bu çalışmada, sağlıklı bireylerde tedavi sonrası amalgamdan zamanla salınan civa miktarının belirlenmesi ve civanın serum MT-1 ve SOD-1 düzeyleri ile ilişkisinin incelenmesi amaçlanmıştır. Gereç ve Yöntem: Yaşları 19-22 arasında değişen 15 deneğe amalgam dolgu uygulandı ve tedavi öncesi ve tedaviden 1 gün, 7 gün, 21 gün ve 35 gün sonra kan örnekleri alındı. Serum numunelerinin civa analizi, İndüktif Olarak Eşleştirilmiş Plazma Kütle Spektrometresi (ICP-MS) kullanılarak yapıldı. Ek olarak, serum numunelerindeki MT-1 ve SOD-1 seviyeleri ticari ELISA kitleri kullanılarak ölçüldü. Elde edilen verilerin analizi Friedman testi ve Spearman korelasyon analizi kullanılarak yapıldı. p<0,05 değeri istatistiksel olarak anlamlılık düzeyinde yorumlanmıştır. Bulgular: MT-1 için yapılan analiz sonucunda değerlerin zamanla azaldığı ve bu düşüşün 21 gün sonra istatistiksel olarak anlamlı olduğu bulundu. Ayrıca SOD-1'in zamanla azaldığı ancak bu düşüşün istatistiksel olarak anlamlı olmadığı bulundu (p>0,05). Salınan civa miktarı açısından zamanla salınan civa değerleri arasında istatistiksel olarak anlamlı bir fark bulunmadı. Korelasyon analizi sonuçlarına göre değişkenler arasında istatistiksel olarak anlamlı bir ilişki bulunmadı. Sonuç: Bu çalışmanın sonuçları, test edilen amalgamdan salınan civa miktarının tolere edilebilir olduğunu ve MT-1 ile SOD-1 arasında anlamlı bir ilişki bulunmadığını göstermiştir. Anahtar kelimeler: Amalgam, antioksidanlar, civa, metallotionein, süperoksit dismutaz*

Ethics Committee Approval: This research has been approved by the Bioethics Committee of the Faculty of Dentistry, Ataturk University (approval number: 20.11.2014/031).

Informed Consent: All subjects included in the study were informed about the study and written informed consent was obtained from the subjects. This study has followed the guidelines stated in the Helsinki Declaration for clinical investigations.

Peer-review: Externally peer-reviewed.

Author contributions: PG, AK participated in designing the study. OK, OS, SA, OT participated in generating the data for the study. OK, OS, SA, OT participated in gathering the data for the study. PG participated in the analysis of the data. PG wrote the majority of the original draft of the paper. PG, OK, OS participated in writing the paper. PG, OK, OS, SA, OT have had access to all of the raw data of the study. PG, AK have reviewed the pertinent raw data on which the results and conclusions of this study are based. PG, OK, OS, SA, OT, AK have approved the final version of this paper. PG guarantees that all individuals who meet the Journal's authorship criteria are included as authors of this paper.

Conflict of Interest: : The authors have no conflicts of interest to declare.

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