Cytotoxicity of Ingredients of Various Dental Materials and Related Compounds in L2- and A549 Cells

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Abstract: Various ingredients of dental materials and related compounds were tested for cytotoxicity in two alveolar epithelial cell lines (L2 and A549 cells). Release of lactate dehydrogenase (LDH) from cells was measured after incubation with the test substances for time intervals up to 48 h and expressed as percentage of total LDH content of lysed cells. Furthermore, the glutathione content of cells was determined in the nonmalignant L2 cells. Additionally, cell viability was assessed by microscopic examination. The highest cytotoxicity was observed with mercury compounds (methylmercuric chloride and mercury dichloride) in the range of 5–20 μmol/l. The composite components 2-hydroxyethylmethacrylate (HEMA) and triethleneglycoldimethacrylate (TEGDMA) showed time- and concentration-dependent effects of cytotoxicity at high concentrations (about 1–5 mmol/l). A time dependence for GSH decrease was mainly found for the composite components up to 12 h of cellular exposure. L2 cells were more sensitive to both mercury and composite compounds than A549 cells. Gold compounds (sodiumaurothiomalate and gold particles < 1.5 μm) did not produce any sign of toxic reactions. A time-dependent increased toxicity in pulmonary cell lines was found for the composite components HEMA and TEGDMA, but not for mercury and gold compounds.
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INTRODUCTION

Toxicity of dental materials has caused public concern in the past years. Composites are discussed as promising alternative to amalgam and the comparatively expensive gold fillings. However, despite allergic reactions,[1–3] the composite materials are described as nontoxic,[4] as well as to exert toxic properties.[5–7]

The lung is exposed to dental materials by various pathways. Amalgam fillings continuously release mercury (Hg) vapor, which may be inhalated.[8] Additionally, ingested Hg is partly absorbed from the gut and reaches the lung by circulation in the blood. 2-Hydroxyethylmethacrylate (HEMA) and triethleneglycoldimethacrylate (TEGDMA) are important composite components occurring in amounts of about 20 to 60% in dental fillings.[9] Both compounds are released from fillings by abrasion or elution and are also known to be inhalated or incorporated by ingestion.[10–12] Moreover, recent results suggest that exhalation is an important pathway of elimination of these compounds.[13] Gold (Au) may also be released from fillings. Here inhalation or exhalation do not occur, but ingestion, absorption, and circulation in the blood is known as well.[14]

Comparative studies on the toxicity of commonly used dental materials on lung cells are not available in the literature so far. The present investigation aimed at assessing cytotoxicity of dental materials and related compounds in two well-established lung cell lines. Both A549 (human, malignant) and L2 (rat, nonmalignant) cells are derived from alveolar epithelial tissue.[15] Lactate dehydrogenase (LDH) leakage and microscopic examination of exposed cell layers were used to assess cytotoxicity because multiple assessment of toxicity from one cell layer is permitted by both of these parameters. The metabolism of the composite components

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tested was postulated to involve radical intermediate products.[16] Therefore the total and oxidized glutathione content of the nonmalignant L2 cells were investigated to assess a parameter directly involved in oxidative processes.

MATERIAL AND METHODS

Chemicals

Cell culture chemicals (DMEMmF12, MEM (Hanks' salts), penicillin/streptomycin and trypsin/N,N,N′,N′-ethylenediaminetetraacetate (EDTA) were obtained from Gibco (Eggenstein, Germany). Fetal calf serum from Biochrom (Berlin, Germany), glutamine, pyruvate, Triton-X-100, $HgCl_2$, and MeHgCl were purchased from Merck (Darmstadt, Germany). HEMA and TEGDMA were from ESPE Dental (Seefeld, Germany), sodiumaurothiomalate (Tauredon®) from Byk Gulden (Konstanz, Germany), and solid gold (particles: 90% $< 1.37\ \mu$m, 50% $< 0.92\ \mu$m, 10% $< 0.63\ \mu$m) from Heraeus (Hanau, Germany). Nicotinamide-adenine dinucleotide (NADH), nicotinamide-adenine dinucleotide phosphate (NADPH), and glutathione reductase (GR) were obtained from Boehringer (Hamburg, Germany), and Coomassie Blue G25 from Serva (Heidelberg, Germany), 5,5′-dithiobis(nitrobenzoic acid) (DTNB) and 2-vinylpyridine from Sigma (Deisenhofen, Germany). All other chemicals and reagents were from various suppliers and were of the highest purity available.

Cell Culture

A549 cells (human, malignant, alveolar epithelial) and L2 cells (rat, alveolar epithelial) were obtained from the American Type Culture Collection (Rockville, MD,). L2 cells were grown in DMEMmF-12 (supplemented with 50 IE/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum) in a moist CO_2 (5% v/v) atmosphere at 37 °C. A549 cells received 5% instead of 10% fetal calf serum. Cells were passaged weekly with the use of a standard trypsin/EDTA protocol[17] with seeding cell densities of about $5 * 10^3$ cells/cm^2 (A549) or $10*10^3$ cells/cm^2 (L2), respectively. Cells were grown for 6–7 days before the experiments were started. Investigations were performed in 24-well dishes; each well had a growth area of about 2 cm^2. The medium was changed every other day.

Exposure Protocol

Confluent L2 and A549 cell layers were washed once with MEM (Hanks' salts) and then incubated with various concentrations of up to 50 mmol/l HEMA, 25 mmol/l TEGDMA, 200 μmol/l MeHgCl, 200 μmol/l $HgCl_2$, 120 μg/ml NaAuM, or 1.2 mg/ml solid gold suspension in DMEMmF12 at 37 °C in 5% (v/v) CO_2 atmosphere for 8 or 24 h, respectively. Additionally, L2 cell layers were incubated for 6, 12, 36, and 48 h with the substances under investigation. All substances were dissolved in growth medium without the use of solvents.

Protein Determination

Protein contents of cell layers were measured according to a modified Bradford procedure.[18] Cells were washed twice with PBS, then incubated in 0.5 N NaOH for 15 h at 37 °C prior to dye-binding. Bovine serum albumin was used as a calibration standard.

Measurement of LDH Release

Following exposure to the substances at the indicated concentrations and time intervals the incubation medium was removed from the cells. LDH activity in the medium was measured according to Vassault.[19] Total LDH activity of control cell layers was determined after lysis with Triton-X-100 (0.1%). This value represented the maximum LDH release from cells (= 100%). All other values were expressed as percentage of this maximum. Experiments were performed with single objects and measured in duplicates. Values were plotted on a concentration log scale and range of maximum slope was detected. Half effective concentrations (EC_{50}) were determined from the ranges of maximum slope.

Microscopic Examination of Cell Layers

Cell layers were examined by microscopic viewing after exposure to the test substances at the indicated concentrations and time intervals. Cellular destruction has been shown by retraction, blebbing, or lysis of cells. Lowest concentrations with clear signs of cellular destruction were noted for every time period of incubation (range is given later in Table II). Photographs were taken with a 10-fold magnification of the microscopic view on an AGFA pan 100 film.

Measurement of Glutathione Content

Total and oxidized glutathione contents of cell layers were measured by the glutathione reductase recycling assay as described by Tietze[20] using DTNB and NADPH. The analytical detection limit was found to be approx. 0.2 nmol glutathione per mg of cellular protein. The method was found to be not influenced by HEMA or TEGDMA, but was influenced by the mercury compounds at $\geq 1\ \mu$mol/l.

For measurement of oxidized glutathione content 2 μl of 2-vinylpyridine were added to 100 μl acidic extract. After the pH was increased to about 7 with the use of triethanolamine, the reaction was allowed to proceed for 1 h at room temperature. Then, excess vinylpyridine was removed by vaporization. Calibration was performed with freshly prepared aqueous GSSG standard solutions.

Calculations and Statistics

Data are presented as means ± standard deviation (SD). Statistical significance of the differences between the experimental groups was checked by the Mann–Whitney rank sum test or student's t-test.

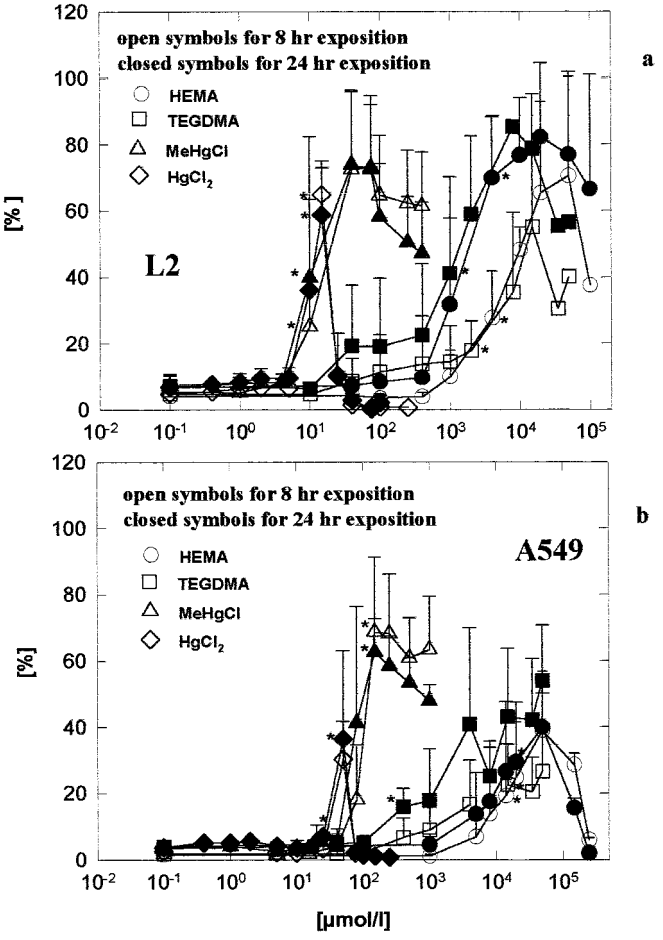

Figure 1. LDH leakage from (a) L2 and (b) A549 cells under the influence of various dental materials and related compounds. Cells were exposed for 8 or 24 h. LDH leakage into the medium is expressed as percentage of total LDH content of lysed cells. Means + SD of four independent experiments are given (*$p < 0.05$, Student's *t* test, first concentration different from control). The investigated gold compounds showed no measurable effects (data not depicted).

RESULTS

LDH Leakage

Total LDH activity of lysed control cells of independent experiments was 0.94 ± 0.1 (L2 cells, $n = 6$) and 1.56 ± 0.26 mU/mg protein (A549 cells, $n = 4$), respectively. Basal LDH leakage of controls amounted to 4.5% and 10.7% of total LDH activity after the 6- and 48-h periods chosen for exposure experiments.

Although the investigated gold compounds (Au, NaAuM) caused no measurable increase in LDH leakage in either cell line, time- and concentration-dependent effects were detected with both mercury and composite compounds. The mercurials were significantly more toxic than the composite compounds in both cell lines.

In L2 cells [Figure 1(a)] both mercury compounds ($HgCl_2$, MeHgCl) increased LDH leakage at concentrations >10 µmol/l after 8 and 24 h of exposure, and HEMA and TEGDMA required concentrations in the millimolar range (>1 mmol/l). A549 cells [Figure 1(b)] were less sensitive. Here >50 µmol/l of $HgCl_2$, > 150 µmol/l of MeHgCl and >14 mmol/l of HEMA or TEGDMA were effective after 8 h of exposure. Slightly lower concentrations of the composite materials were needed after 24 h of exposure.

Table I summarizes the EC_{50} values calculated for the effects in L2 cells of an additional series of experiments performed with exposure times of 6, 12, 36, and 48 h. EC_{50} values for HEMA decreased with exposure times from about 5 mmol/l after 6 h to about 3 mmol/l after 36 h and to 0.7 mmol/l after 48 h. TEGDMA was slightly more effective with a decrease from 3 mmol/l (6 h), to 1.5 mmol/l (24–36 h) and 0.6 mmol/l (48 h). With MeHgCl and $HgCl_2$ EC_{50} values decreased from about 18 µmol/l to 6 µmol/l and 35 to 4 µmol/l, respectively.

Microscopic Examination of Cell Layers

Cytotoxicity as assessed by microscopic examination revealed similar results as obtained in the LDH leakage experiments (Table II). Accordingly, no visible signs of cytotoxicity were expressed in layers of both cell lines exposed to solid gold suspensions and solutions of the tested organic gold compound (NaAuM) (Table II). Again, the other compounds were more toxic in L2 cells than in A549 cells; the first changes indicating detrimental cellular reactions (like rounding, blebbing, lysis) were generally observed at lower concentrations/shorter exposure intervals (data not shown).

Figure 2 shows a typical picture of a damaged L2 cell layer in comparison to control cells. In Table II the noted

TABLE I. EC_{50} Values for LDH Leakage from L2 Cells after Exposure to Various Dental Materials and Related Compounds. Cells Were Exposed to Nine Different Concentrations of Every Compound. At Indicated Time Intervals Medium was Removed for LDH Measurement. Values are Expressed as Percentage of Total LDH Content. EC_{50} Values of Four–Six Independent Experiments were Averaged and Means ± SD Are Given

	6 h	12 h	24 h	36 h	48 h
HEMA [mmol/l]	5.0 ± 2.4	4.3 ± 2.4	3.3 ± 2.1	2.5 ± 2.7	0.7 ± 0[a]
TEGDMA [mmol/l]	3.0 ± 3.0	2.0 ± 2.5	1.7 ± 0.6	1.7 ± 0.6	0.6 ± 0.8
MeHgCl [µmol/l]	18.2 ± 8.7	7.3 ± 3.3	8.7 ± 0	8.7 ± 0	6.3 ± 3.8[a]
$HgCl_2$ [µmol/l]	36.2 ± 35.4	67.8 ± 37.5	60.2 ± 37.8	21.4 ± 26.6	3.6 ± 2.4
NaAuM	nc[b]	nc	nc	nc	nc
Au	nc	nc	nc	nc	nc

[a]Significantly different from 6-h exposure period, $p < 0.05$.
[b]nc—not calculated, because no increase occurred.

TABLE II. Threshold Concentration for Toxic Signs Determined by Microscopic Examination. Cells Were Exposed to Nine Different Concentrations of Every Compound and Examined after the Indicated Time Intervals. Lowest Concentration with Clear Signs of Damage Was Noted. The Range of at Least Three Independent Experiments Is Listed

	6 h	12 h	24 h	36 h	48 h
HEMA [mmol/l]	> 5	1–5	1–5	1–5	0.4
TEGDMA [mmol/l]	> 4	0.4–4	0.4–4	0.4–1	0.4
MeHgCl [μmol/l]	15	15	15	15	5
$HgCl_2$ [μmol/l]	25	25	25	25	25
NaAuM	ns[a]	ns	ns	ns	ns
Au	ns	ns	ns	ns	ns

[a]ns = No signs of toxic reaction by microscopic examination.

concentrations with first clear signs of cytotoxicity in L2 cells after various times of exposure to the test compounds are listed.

With HEMA the threshold concentration for toxic microscopic signs was 5 mmol/l at exposure periods up to 24 h. This concentration decreased to 1–5 mmol/l after 36 h and to 0.4 mmol/l after 48 h of exposure. With TEGDMA the first changes were seen at 1–4 mmol/l after an exposure period of 6 and 12 h. The threshold decreased to 0.4–4 after 24 h, to 0.4 –1 mmol/l and 36 h and to 0.4 mmol/l after 48 h of exposure.

MeHgCl caused first signs of destruction at 15 μmol/l after 6–36 h and at 5 μmol/l after 48 h of exposure. Incubation of cells with 25 μmol/l $HgCl_2$ was followed by toxic signs at all exposure times examined.

Glutathione Content of L2 Cells

After exposure periods of 6 h decrease in GSH content was too low to fit IC_{50} values. The GSH contents begun to decrease after exposure of cells to about 0.1 mmol/l HEMA or TEGDMA for 12 h. When exposure periods were prolonged this threshold concentration was slightly decreased. IC_{50} values for the different exposure periods are given in Table III. A small increase in toxicity was found after prolonging the exposure periods. IC_{50} values for GSH depletion were clearly below values found for LDH leakage of morphological signs of toxicity for exposure periods of 12 h or above. No increases in GSSG contents by HEMA or TEGDMA exposure were found (not shown).

After cells were exposed to the mercury compounds a decrease in cellular GSH and an increase in GSSG was found. IC_{50} values of GSH depletion are given in Table III. Values are in the range of those for LDH release and microscopically detected toxic signs.

DISCUSSION

In the present study the toxicity of various dental materials and related compounds was compared in lung cells. The two cell lines used reveal characteristic properties of the alveolar epithelium. Both are known to contain enzymes needed for surfactant synthesis.[21] Additionally, both cell lines reveal typical inclusion bodies.[15] The human-derived A549 line is of malignant origin and was initiated in 1972 through explant culture of lung carcinomatous tissue.[15] The L2 cell line,

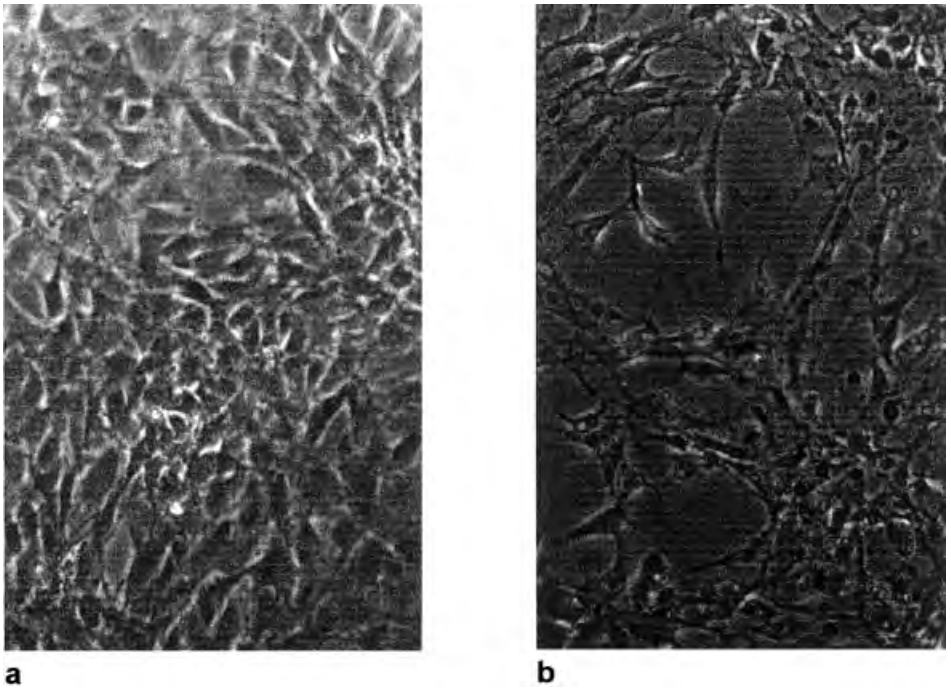

Figure 2. Microscopic examination for cytotoxicity in L2 cells. (a) Control cell layer; (b) cell layer after exposure to 8 mmol/l HEMA for 24 h (about 300-fold magnification).

TABLE III. IC_{50} Values for GSH Depletion in L2 Cells after Exposure to Various Dental Materials and Related Compounds. Cells Were Exposed to Seven Different Concentrations of Every Compound. At Indicated Time Intervals Cells were Denaturated with Perchloric Acid and GSH and GSSG Were Determined as Described. IC_{50} Values of GSH Depletion of Four Independent Experiments Were Averaged, and Means ± SD Are Given.

	6 h	12 h	24 h	36 h	48 h
HEMA [mmol/l]	> 8.0[a]	0.49 ± 0.34	0.28 ± 0.15	0.14 ± 0.07	0.13 ± 0.07
TEGDMA [mmol/l]	> 3.0[a]	0.23 ± 0.11	0.15 ± 0.06	0.14 ± 0.03	0.12 ± 0.05
MeHgCl [μmol/l]	13[b]	20[b]	22[b]	2[b]	1[b]
$HgCl_2$ [μmol/l]	17[b]	20[b]	19[b]	12[b]	6[b]

[a]GSH depletion below to fit EC_{50} values, highest concentration tested is given.
[b]Values observed are in the range of toxic effects and substance tested influences the determination method; values therefore might be determined as too low; no SD is given.

however, is nonmalignant and was derived from enzymatically dispersed adult rat lung.[15]

As expected, among the metal compounds tested the Hg derivatives were much more toxic than Au compounds in both cell lines. Neither metallic Au nor NaAuM showed any cytotoxic effects, whereas both $HgCl_2$ and MeHgCl were clearly cytotoxic in the micromolar range. However, L2 cells were more sensitive than A549 cells to either Hg derivative. This can be explained by metabolic differences between the two cell lines. Malignant cells usually contain much more glutathione (GSH) than other cells. Accordingly, the GSH content of A549 cells is about 10 fold higher than that of L2 cells.[22] Additionally, higher activity of the GSH regenerating enzyme glutathione reductase (GR) is found in A549 cells.[23] GSH is thought to be the first step in detoxification (by affinity association) of SH reactive metals like Cd, Hg,[24] but even As, Zn, Cu, Co, Ni, Pb, and others.[25] Therefore the increased toxicity of the mercury compounds in L2 cells was expected. Over all the higher fcs content in the investigations with L2 cells may not explain the enhanced sensitivity of these cells. Fcs is known to reduce free concentrations due to unspecific binding sites, therefore diminishing the toxic potential of substances. But in L2 cells with the higher fcs contents an increase in toxicity was found.

In L2 cells a time-dependent decrease of the toxic threshold concentrations for mercurials was found by about threefold for MeHgCl and by about tenfold for $HgCl_2$ from 6 h up to 48 h of exposure. The higher cytotoxicity of MeHgCl compared to $HgCl_2$ may be explained by the lipophilic character of MeHgCl. Klaassen described a higher cellular incorporation rate of the more lipophilic organic mercury compound as compared to the inorganic hydrophilic $HgCl_2$.[26]

Compared to mercurials the composite components TEGDMA or HEMA revealed much lower cytotoxicity. This was evident by LDH leakage in concentrations of a milimolar range (Table I). HEMA seems to be less toxic than TEGDMA. This has been found by other authors as well.[5,7,27] Comparing the toxic concentrations this milimolar range was found by Ning[28] for HEMA as well, while other authors often found toxic effects in a range of 0.1–1 mmol/l for the substances tested.[5,29] This possible rudeness of the present results may be caused by the choice of the toxicity assay, as LDH leakage is known to be affected late. Furthermore it is known that other cytotoxicity assays, such as dye exclusion or dye inclusion tests, overestimate cellular viability[17] and especially in the case of long-term effects avital cells might be deleted from the cell layers and therefore cannot give signals of lethality or viability. A further advantage of measuring LDH leakage is the possibility of the kinetic properties due to this method.

IC_{50} values for GSH depletion by the composite components are much lower than for LDH leakage. Values are in the range of 0.1–1 mmol/l as described for toxicity by Geurtsen et al. or Lefebvre et al.[5,29] . As GSH depletion often is used as a marker of toxicity one might give about 0.15 mmol/l as the lowest toxic concentration for the composite components in this study after cellular exposure of 48 h. But one has to consider that GSH content may not be a good parameter of toxicity, while a decrease in the ratio of GSH/GSSG may indicate toxicity very well. No increase in GSSG was found in this study by the composite components tested, whereas the mercury compounds clearly increased cellular GSSG contents. It is assumed that GSH depletion by composite components may be caused by an increased GSH consumption that is partly (but sufficiently) compensated and therefore might not reflect a toxic incident. GSH depletion by TEGDMA was found to occur in 3T3 fibroblasts after 24 h of incubation at comparable concentrations as in this study.[29] Therefore it is concluded that the sensitivity of our lung cells toward the composite components tested is not different as compared to other cell lines.

However, both cell lines demonstrated a clear time dependence in the toxicity of HEMA and TEGDMA with an about tenfold (and fivefold resp.) decrease of the toxic concentration as measured by LDH release during 6 and 48 h of exposure. Interestingly, again L2 cells were more sensitive than A549 cells. An explanation might be that it is not the composite components themselves, but the time-dependent formation of toxic metabolites leading to oxidative stress and thereby enhanced GSH consumption that is responsible for the observed effects. This was postulated by Durner et al.[16] In accordance with that GSH depletion by the composite components tested took place between the 10th and 14th hour of cellular exposure. However, further studies are necessary to confirm this hypothesis of toxic metabolites.

In the LDH leakage experiments increased enzyme activities measured in the medium above exposed cells showed a recurrent trend at very high concentrations of both mercurials

and composite materials. This was probably due to an artifact, for example, action of high concentrations of the substances on the enzyme itself. Microscopic examination showed no changes (recurrent trends) in cytotoxicity at high concentrations.

This effect points to a further methodological topic: the influence of mercury compounds on the measurement of glutathione. It was found that about 1 μmol/l $HgCl_2$ or MeHgCl reduced the GR activity partially and about 2.5 μmol/l inhibited activity completely. As the mercury content of the cells used here is not known, the possibility that the glutathione contents measured might be affected by this methodological artifact cannot be excluded. It has to be considered that the glutathione content might be measured as too low. But in such cases the total cellular GR activity also has to be described as totally inhibited. Such a cellular situation would be lethal. Therefore if glutathione contents were measured in the range of lethal mercury concentrations (as given in Tables I and II) those contents have to be judged very carefully. On the other hand, values given are in an expected range. Furthermore, because mercury compounds are known to inhibit GR, an expected effect is the GSH depletion accompanied by an GSSG increase and a subsequent lethality. Such a sequence of events might be reflected by the present data.

Because of the artificial conditions in the studied cell culture model, one has to be careful in drawing any conclusions concerning a possible toxicological risk posed by the studied compounds in vivo. However, considering that the average mercury content in the blood of people with amalgam tooth fillings (>15) is about 4 μg Hg/l blood = 0.02 μmol/l Hg/l blood, and that in the studied cells the first toxic effects occurred above > 1 μmol/l (factor 50 higher), a risk of acute toxic lung cell damage by Hg released from amalgam fillings is unlikely. Although at present, a similar evaluation is not possible for composite compounds because of a lack of pharmacokinetic data, due to the comparatively low cytotoxicity found in the present experiments, a pulmononary toxicological risk by dental composite components seems even more unlikely.

Abbreviations used in this article are as follows: DMEMmF12, Dulbecco‘s modified Eagle medium/Ham‘s F 12 nutrient mix (1:1); DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EC_{50}, concentration for half maximal effect; EDTA, N,N′,N′-ethylenediaminetetraacetate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HEMA, hydroxyethylmethacrylate; $HgCl_2$, mercuric chloride; LDH, lactate dehydrogenase; MeHgCl, methylmercury chloride; MEM, minimum essential medium; NaAuM, sodiumaurothiomalate; TEGDMA, triethyleneglycoldimethacrylate.

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