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ORIGINAL ARTICLE

Uncompleted polymerization and cytotoxicity of dental restorative materials as potential health risk factors

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Abstract

Introduction. Composite materials used in dentistry indicate adverse biological effects in laboratory conditions. One reason for this activity is incomplete conversion of their polymer matrix, favoring chemical instability and release of biologically harmful components to the external environment.

Aim. The aim of the study was to assess the degree of conversion of restorative materials commonly available on the European market and to examine the cytotoxic effects of their eluates in vitro.

Material and methods. Using the Fournier transform infrared spectroscopy (FTIR) technique of analysis, the degree of polymer matrix conversion of 6 restorative materials was examined: Gradia Direct, Arkon, Filtek Z550, Herculite XRV, Tetric Evo Ceram, Charisma, polymerized with LED light. In order to assess the cytotoxicity of eluates of the studied materials obtained after 1 hour, 24 hours and 7 days, the MTT assay was used in cultured 3T3 cells. The results were statistically analyzed at significance level of p=0.05.

Results. The conversion degree of the assessed polymers ranged from 31.56% for Tetric Evo Ceram to 75.84% for Arcon. The strongest (p=0.05) cytotoxic effect was demonstrated after 7-day observation of Tetric Evo Ceram eluates, reducing the metabolic activity of cells down to 56%. A positive correlation (r(x, y)=0.62) between the degree of conversion of composite materials and cytotoxic effects of their eluates on cell cultures was confirmed.

Conclusions.

1. Restorative dental materials are chemically unstable in the conditions of the present study.
2. Polymer-based restorative dental materials available on the European market demonstrate cytotoxic properties constituting a potential threat to the patients' health.

Key words

dental composites, cytotoxicity, degree of conversion

INTRODUCTION

Composite materials used in many areas of modern dentistry are made of organic matrix, inorganic filler, coupling agent/silane and systems of initiators, catalysts and polymerization inhibitors. Matrix of polymer-based composites consists of basic monomers Bis-A, UDMA, and comonomers such as TEDGMA, EDGMA and HEMA [1]. During polymerization, monomer and comonomer molecules merge to form a spatial network connected to an inorganic filler fraction. Polymerization of most restorative materials is initiated by visible light emitted by lamps. Unfortunately, numerous studies indicate that the cross-linking process of composite materials is incomplete [2]. Incompletely polymerized material contains partially unbounded monomers released directly to the external environment [3]. Incompletely polymerized non-homogeneous material is also more susceptible to physical and chemical degradation [4, 5]. To-date, about 30 [6] chemical compounds emitted from dental materials have been identified, including biologically harmful ones such as TEGDMA, UDMA, EDGMA, HEMA or bisphenol A. Released components of composite materials have cytotoxic [7], mutagenic [8] properties, and they can be potent allergens [9]. Nowadays, research teams pay special attention to bisphenol A, a cytotoxic compound with biological activity of estrogen group hormones. The wide spectrum of its adverse effects on living organisms is confirmed in many studies. BPA may be responsible for impaired spermatogenesis, oogenesis, of causing disorders of nervous system development in fetuses, and the induction of gland cell cancerous hypoplasia. Thus, the safety of polymer-based dental materials releasing BPA raises doubts, particularly in the treatment of pregnant women.

The conversion degree of polymerized materials determines their stability and depends on many factors, including the chemical structure of monomers, effectiveness of photoinitiators, filler type, translucency of material, thickness of the irradiated layer, distance between the light source and

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polymerized material, light intensity and emission time, and the composition of the surrounding atmosphere [10]. The presence of oxygen-containing air in the oral cavity contributes to the creation of an oxygen inhibition layer on the surface of composites, where the polymerization process occurs to a low degree [11]. Laboratory tests offer optimal conditions for polymerization, thus the values of conversion in the laboratory may be higher than those observed in clinical conditions.

The literature reports many different methods for assessing the cytotoxic effects of dental materials. They include tests for estimating the amount of ribonucleic acids and the damage to their chains [12], assessing the glutathione level in cells [13], assessing the expression of heat shock proteins [14], or studies evaluating the severity of apoptotic action [15]. Each of these methods has advantages and disadvantages, but provide extra information that cannot be obtained by other methods.

The cytotoxicity of dental materials is commonly tested by evaluating the effect of the studied materials' eluates on cultured cells. These methods allow imitation of oral cavity conditions, where dental composites remain in constant contact with saliva or fluids consumed by patients, which act as media for potentially harmful biological substances. A common method used to assess the cytotoxic activity of dental materials in tissue cultures is the MTT assay [16, 17]. This test utilizes the ability of an enzyme contained in the mitochondria of living cells to catalyze the reduction reaction of thiazolyl blue formazan to insoluble formazan. The reduced compound is red, and its concentration in the culture is expressed by colour change, which is evaluated using a spectrophotometer. The cells most commonly used in this method are gingival fibroblasts, keratinocytes in oral epithelium, and standardized strains of mouse L -929 or 3T3 fibroblasts.

OBJECTIVE

The aim of the study is a comparative assessment of the conversion degree of dental restorative materials, and evaluation of cytotoxicity of their eluates obtained after storage in water.

MATERIALS AND METHOD

Six restorative materials were evaluated: Gradia Direct Anterior (GC Corp., Japan), Arkon (Arkona, Poland), Filtek Z550 (3M, USA), Herculite XRV (Kerr Italia, Italy), Tetric Evo Ceram (Ivoclar-Vivadent, Liechtenstein), Charisma (Haraeus Kulzer, Germany). The assessed composites were obtained from Polish distribution sources and have been approved for sales in the EU (Tab. 1).

Assessment of conversion degree of composite materials. To measure the conversion degree of the assessed composite restorative materials, the Fourier Transform Infrared Radiation analysis was used with a Nicolet IS 10 (Thermo Scientific, USA) spectrometer with a type II diamond crystal Smart Orbit accessory (Fig. 1). The studied composites were placed in Teflon matrices 5 mm in diameter and 2 mm deep. They then were removed from the matrices and placed on the diamond crystal of the accessory. Subsequently, the spectrum of infrared radiation reflected off the test material was recorded. After that, the materials were removed from the crystal and placed between two layers of polypropylene film and formed with Teflon plates, obtaining a 1 mm-thick layer. After removing the plates, the studied material was polymerized by LED 55 curing light (TPC Advanced Technology, USA) at 1,100 mW/cm^2 for 20 seconds. When polymerization was complete, the material samples were removed from the matrices and stored in darkness. After 24 hours, another analysis of infrared spectrum reflected off

Figure 1. Nicolet IS10 Spectrometer (Thermo Scientific, USA)

Table 1. Restorative materials evaluated in the study

dental composite	manufacturer	country of origin	serial number	expiry date	shade	harmful substances according to the manufacturer declaration
Gradia Direct Anterior	GC Corp.	Japan	1104113	2014-04	A3	UDMA, CQ
Arkon	Arkona	Poland	20110401	2013-03	A3	Bis-GMA, UDMA, TEDGMA
Filtek Z550	3M	USA	N320181	2014-07	A3	Bis-GMA, Bisphenol Adi-(2-hydroks yetylo- etero) di-metakrylan, UDMA
Herculite XRV	Kerr Italia	Italy	3653864	2014-03	A3	estry metakrylanu
Tetric EVO Ceram	Ivocrar Vivadent	Lichtenstein	N34600	2014-05	A3	Bis-GMA, UDMA
Charisma	Haraeus Kulzer	Germany	010301	2012-09	A3	metakrylany

the material was performed. The method was used to assess five samples of each material. An example of spectral weighting of radiation reflected off assessed sample material is shown in Figure 2.

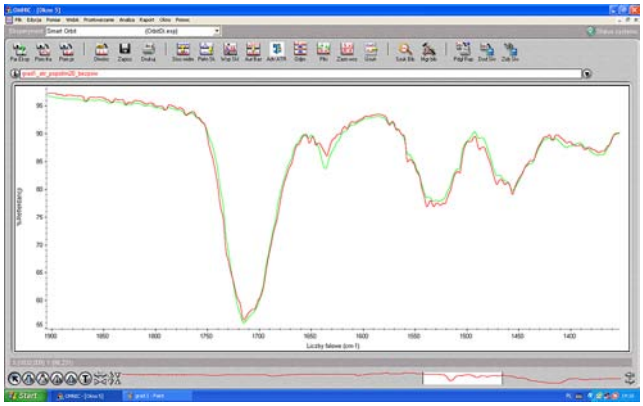

Figure 2. An example of spectral weighting of radiation reflected from assessed sample material

Assessment of cytotoxicity of eluates from the studied restorative materials. In order to obtain eluates, five samples of each studied material were prepared, which were then placed in Teflon matrix hollows 5 mm in diameter and 2 mm deep, covered with a polypropylene film, and then polymerized with LED 55 curing light for 20 seconds. After polymerization, the material was removed from the matrix and stored in darkness for 24 hours at 25 °C. The collected samples were placed in glass vials filled with 10 mL of HPLC grade water containing 0.05 ml of Antibiotic Antimicotic (Invitrogen, USA). Five samples of each studied material were incubated at 37 °C for 1 hour, 24 hours and 7 days, and then removed from the vials. The obtained aqueous eluates were used for evaluating the cytotoxicity of composite materials. Cytotoxicity assessment was performed with the use of succinate dehydrogenase activity assay/MTT assay in mouse fibroblast cultures of standardized Balb/c 3T3 cell line. The studied eluates buffered with 0.9% NaCl, were applied to the culture in six replications. Aqueous control solutions contained a poly-antibiotic mix buffered with saline. The cells were incubated with eluates of the studied materials and with control solutions for 60 minutes; the fluids were then removed from the cells. The plates with cell culture were loaded with 100 µl of DMEM (Dulbeco Minimal Essential Medium) containing 50 µl thiazolyl blue tetrazolium bromide solution (Sigma- Aldrich, USA) in PBS solution (Invitrogen, USA). The studied 3T3 cell cultures were incubated for a further 3.5 hours, then the medium was removed and the resulting formazan crystals dissolved in 100 ml of dimethyl sulfoxide (Sigma-Aldrich, USA). Using a Multiskan RC reader (Thermo Fisher Scientific, USA), the absorbance of light at 540 nm and 650 nm wavelengths was determined. The number of cells surviving incubation with eluates of the studied composites was expressed in percentage proportion to the number of cells in the control cultures (100%).

Statistical analysis. The results were statistically analyzed at the assumed significance level of p = 0.05. For continuous variables, the following were calculated: numbers, arithmetic mean, standard deviation, median, minimum value and maximum value.

The basic tool in the average analysis was the model of one-way analysis of variance (ANOVA one-way). Normal distribution was assumed. The assumption of equality of variance was tested with the Brown-Forsthe test. Tukey test was used for multiple comparison. In order to investigate the correlation between the conversion degree of the assessed composites and cytotoxic effects of their eluates, the Pearson method of evaluation was used.

RESULTS

Conversion degree of studied composites. The conversion rate of the assessed materials 24 hours after polymerization of samples with visible light emitted by LED lamp ranged from 31.56% for Tetric Evo Ceram to 72.12% for LC Contex, averaging 56.09 %. Statistical analysis at p = 0.05 significance level showed that Tetric Evo Ceram and Charisma materials were cross-linked to a significantly lower degree than the other polymers. Significantly (p = 0.05), the most cross-linked composite was Arkon, for which the average conversion degree equaled 75.84 %.

The conversion degree of the assessed restorative materials is shown in Table 2.

Table 2. Comparison of conversion degree of assessed materials for filling hard tissues of teeth

material	degree of conversion	SD	comparison of values p=0.05			
			1	2	3	4
Tetric Evo Ceram	31.56	5.06	****			
Charisma	35.92	8.56	****			
Filtek Z550	39.34	12.27	****	****		
Gradia Direct Ant.	50.99	7.49	****	****	****	
Herculite XRV	66.70	7.66			****	****
Arkon	75.84	17.93				****
mean	50.06	9.83				

Cytotoxicity of eluates of studied composites. The average viability of 3T3 fibroblasts exposed to eluates obtained after one-hour storage of composites in water equaled 96.2%, not differing significantly (p>0.05) from the viability of cells treated with control solutions.

Tetric Evo Ceram and Filtek Z550 eluates significantly (p<0.05) reduced the metabolic processes in cell cultures, down to 86.8% and 92.2%, respectively, compared to the 100% of the control cultures.

The metabolic activity of fibroblasts exposed to solutions obtained after 24-hour storage of composite samples in water, on average, was 85.4%, differing significantly (p<0.05) from the control group value of 100%. Eluates of Direct Gradia Anterior, Filtek Z550 and Tetric Evo Ceram, reduced metabolism in cultured cells significantly more strongly (p<0.05) than the other solutions, down to 76.8%, 81.5% and 65.9%, respectively, compared to 100% for the control group.

Eluates obtained after 7 days of sample storage in water significantly (p<0.05) reduced metabolic processes of the cells down to 84.2%, on average, (control culture = 100%). Fibroblast metabolism intensity was affected significantly more strongly (p<0.05) by Gradia Direct Anterior, Filtek Z550 and Tetric Evo Ceram than by solutions obtained from the other materials. The eluates of the mentioned materials

reduced the metabolic processes of 3T3 fibroblasts down to 68.3%, 83.4% and 56.2%, respectively.

The numerical values describing the intensity of metabolism in cells exposed to eluates, obtained after 1 hour, 24 hours and 7 days of observation, are shown in Tables 3, 4 and 5.

Table 3. Numerical values describing intensity of metabolic processes of cells exposed to eluates of composite materials for filling hard tissues of teeth obtained after 1 hour of observation in relation to the control group (100%)

material	vitality of 3T3 cells in relation to control (100%)	SD	p=0.05
Gradia Direct Ant.	98.6	8.4	0.9939
Arkon	99.1	9.1	0.9109
Filtek Z550	92.2	4.5	0.0168
Herculite XRV	105.2	17.7	0.4033
Tetric Evo Ceram	86.8	2.2	0.0000
Charisma	95.1	15.9	0.6119
mean	96.2	11.9	0.2182

Table 4. Numerical values describing the intensity of metabolic processes of cells exposed to eluates of composite materials for filling hard tissues of teeth obtained after 24 hours of observation in relation to the control group (100%)

material	vitality of 3T3 cells in relation to control (100%)	SD	p=0,05
Gradia Direct Ant.	76.8%	7.7%	0.0010
Arkon	90.5%	15.8%	0.2612
Filtek Z550	81.5%	6 %	0.0009
Herculite XRV	98 %	16.1%	0.9281
Tetric Evo Ceram	65.9%	6.3%	0.0001
Charisma	99.7%	10.3%	0.8199
mean	85.4%	16 %	0.0000

Table 5. Numerical values describing intensity of metabolic processes of cells exposed to eluates of composite materials for filling hard tissues of teeth obtained after 7 days of observation in relation to the control group (100%)

material	vitality of 3T3 cells in relation to control (100%)	SD	p=0.05
Gradia Direct Ant.	68.3%	5.5%	0.0000
Arkon	102.9%	7.3%	0.2255
Filtek Z550	83.4%	4.6%	0.0005
Herculite XRV	101 %	19.4%	0.7891
Tetric Evo Ceram	56.2%	5.4%	0.0000
Charisma	93.4%	10.9%	0.2820
mean	84.2%	19.8%	0.0001

Statistical analysis at the significance level of p=0.05 showed that eluates obtained after 24 hours and 7 days of storage in water reduced the metabolic processes of 3T3 cells significantly more strongly ($p<0.05$) than the solutions obtained after 1 hour of incubation.

Assessment of correlation between conversion degree of polymer-based restorative materials and their eluates' effect on metabolic processes of 3T3 fibroblasts. Analysis of the results performed by the Pearson method showed a positive correlation ($r_{(x, y)} = 0.62$) between the conversion degree of the studied materials and cytotoxic effects of their eluates on metabolic processes of cells in tissue cultures.

DISCUSSION

The measured values of the conversion degree of materials assessed in the presented study, ranging from 31.56% – 75.84%, are comparable with the results of other authors assessing the degree of cross-linking of dental composites.

In a study by da Silva et al [18], the authors assessed the conversion degree of Filtek Supreme (3M, USA), polymerized by halogen light for 20 – 30 seconds. The authors used the FTIR method, as in the presented study. They prepared composite samples in a similar way, forming thin (approximately 60μm) layers for reflection spectrometry. The authors observed the conversion degree of the material at 49–53%, which is within the range of values obtained for the materials examined in the current study, although the quoted authors used different light sources and exposure time during polymerization.

FTIR was also used by Schneider et al. [19] assessing the conversion rate of Filtek Z250 polymerized by LED light for 20 seconds. Despite different sample preparation methods and different composite compared to the presented study, the degree of conversion of Filtek Z250 amounted to 49.32%, which again was within the range of values obtained in the current study for restorative materials.

Tarle et al. [20] evaluated the conversion degree of Tetric Ceram (Ivoclar Vivadent, Liechtenstein) polymerized by LED light for 40s. The quoted authors used Fournier transform infrared spectroscopy, the same as in the presented study. According to Tarle et al. [20], the conversion degree of Tetric Ceram equaled 61% and was almost double that of the Tetric Ceram conversion observed in the current study.

In a study by Rojas et al. [21], the conversion degree of the same material was measured. The authors polymerized material samples with LED light for 40s and then assessed the cross-linking degree using FTIR. The authors reported the conversion degree of Tetric Ceram at 63%, which is twice higher than that observed in the presented study. The values reported by Rojas et al. [21] were similar to the results obtained by Tarle et al. [20].

Perhaps the divergence between the results of Tarle et al. [20] and Rojas et al. [21], on the one hand, and the results of this study on the other hand, was due to different sample preparation methods, different testing equipment and the polymerization protocol applied by the authors.

Soares et al. [22] evaluated the degree of conversion of Charisma after polymerization using a variety of curing lamps and exposure protocols. Depending on the sample preparation protocol, the authors reported a cross-linking degree of the material of from 46–57%. In the presented study, the conversion degree of Charisma was under 36%, and was lower than the value observed by Soares et al. [22]. Here, too, different methodology could contribute to the discrepancies between the results of both studies.

Cytotoxicity of composite restorative materials was studied also by Al-Hiyasat et al. [16]. The authors polymerized Filtek Z250 and Tetric Ceram samples with LED light, and after 24 hours of storage placed them directly in 3T3 fibroblast cultures suspended in 1 ml medium for another 72 hours. Al Hiyasat et al. [16] assessed the level of metabolic processes in the cultures using MTT assay, the same as in the presented

study. Direct contact of the culture with Filtek Z250 and Charisma materials reduced the metabolic processes of the cells down to about 60%, compared to 100% of the control group. In the presented study, Charisma eluates reduced the metabolic processes down to 95.1%, 99.7% and 93.4%, compared to control cultures, depending on the duration of sample incubation in the aqueous environment. In both studies, samples of similar volume were used, as well as MTT assay and 3T3 fibroblast cell lines. The lower cytotoxicity of Charisma observed in the presented study compared to the value reported by Al Hiyasat et al. [16] can be explained by the 10-fold larger volume of fluid used for sample stoprae in the presented study, and by the fact that Al Hiyasat et al. placed material samples in cell cultures where cytotoxic substances were directly released.

Beriat et al. [17] evaluated the cytotoxicity of Filtek Z250 and Filtek Supreme materials by performing MTT assay in L-929 fibroblast line cultures. Material samples were polymerized with halogen or LED light and placed directly in cell cultures. After 8 hours of incubation, both materials reduced the viability of L-929 cells down to about 80%, compared with 100% of the control culture, regardless of the applied polymerization protocol. Although the reduction in cell metabolic processes reported by the authors was similar to that obtained in the presented study, it was not possible to directly compare the results of both studies due to different incubation time of the samples, differences in their volume, direct contact of materials with cultured cells, and unknown volume of culture medium in which the samples were stored.

Cytotoxicity of Filtek Z250, Tetric Ceram and Herculite HRV materials was assessed by Franz et al. [11] using MTT assay performed in L-929 fibroblast cultures. Samples were polymerized with halogen light and then transferred into cell cultures. Materials introduced into the culture immediately after irradiation reduced the metabolic processes down to 75–87%. Samples introduced after 72 hours reduced cell viability down to 90–99%, which is close to the values obtained in the presented study for eluates obtained after 1 hour of sample storage in water. Although Franz et al. [11] prepared samples of materials similar in volume to those assessed in the presented study, and in the case of Herculite HRV the same material was examined, it was not possible to directly compare the results of both studies due to different methods of evaluation.

The results of the current study indirectly support the thesis that restorative materials gradually degrade with time, releasing more and more cytotoxic components. This is confirmed by a significantly higher ($p<0.05$) reduction in the metabolic processes of cells obtained after 24 hours and 7 days of storage of the materials in water, compared with eluates obtained after 1 hour of sample incubation. Unfortunately, the literature offers no studies that could allow tracing changes in cytotoxicity of eluates from restorative materials in time, and to compare their results directly with the results of the presented study.

Despite the widely accepted assumption that an increase in the conversion degree of a composite material favours its chemical stability and reduces the release of potentially harmful chemical compounds, in the literature there are no studies directly evaluating correlations between the degree of conversion of composites and their cytotoxic effects on metabolic processes of cells in *in vitro* conditions.

In a study by Jagdish et al. [23], the authors analyzed the correlation between the degree of conversion of orthodontic adhesives, structurally close to restorative materials, and their cytotoxic effects *in vitro*. The quoted authors reported no significant association between the conversion degree of the material and impact of its samples on the intensity of metabolism in cultured cells.

The presented study demonstrates a positive correlation between the conversion degree of dental materials and their cytotoxic effects on 3T3 cells. The correlation supports the concept that the biologically harmful effects of restorative dental materials depend on the degree of polymerization of their organic matrix, which improves their chemical stability.

CONCLUSIONS

The quoted results and the results of the presented study confirm the thesis of insufficient effectiveness of polymerization of dental composites and of their cytotoxic impact on cell cultures. Therefore, it is necessary to continue research in order to improve this medical product group, and monitor their safety by means of standardized assessment methods allowing comparisons of research results.

Due to increasing reports on the harmful effects of chemical compounds contained in dental materials on developing organisms, particular attention should be paid to the safety of restorative composites for pregnant women and young children.

Restorative dental materials are chemically unstable in the conditions of the present study, and the polymer-based dental materials present on the European market exhibit cytotoxic properties which constitute a potential threat to the health of patients.

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