Original Paper

The Cytotoxicity of Dental Restorative Materials on Gingival Stromal Mesenchymal Cells-an *In Vitro* Study

DIANA ONEȚ[1], ALEXANDRA ROMAN[1], ANDRADA SOANCĂ[1], ANDREEA CIUREA[1], IULIA CRISTINA MICU[1], ȘTEFAN CRISTIAN VESA[2], DORA-MARIA POPESCU[3], ANA-MARIA RÎCĂ[4]

[1]*Department of Periodontology, „Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

[2]*Department of Pharmacology, Toxicology and Clinical Pharmacology, „Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

[3]*Department of Odontology, University of Medicine and Pharmacy of Craiova, Romania*

[4]*Department of Periodontology, University of Medicine and Pharmacy of Craiova, Romania*

ABSTRACT: Background: Due to their minimally invasive high-quality adhesive, aesthetic and mechanical qualities, composite resins are the most frequently used materials in modern restorative dentistry. However, polymerization shrinkage and cytotoxicity are still unresolved drawbacks associated with these biomaterials. Purpose: The present study aimed to assess the cytotoxicity of some restorative resin-based materials on gingival mesenchymal stromal cells (gMSCs), assuming that no differences in their behavior will be highlighted. Material and methods: The cytotoxicity of the tested materials was evaluated by comparing the behavior of gMSCs cultured in normal conditions and in association with disc-shaped material samples indirectly through functionality tests (colony-forming unit-fibroblast assay, migratory potential) and directly through the MTT assay. The results were statistically analyzed with the ANOVA test and Tukey's Honest Significant Difference test. Results: According to the MTT assay, there are no statistically significant differences regarding the viability of gMSCs cultured in normal conditions or in the presence of resin-based material samples. On the other hand, the present study identified a significantly reduced number of colonies formed by the gMSCs cultured in association with BF and B discs, compared to that of gMSCs cultured in normal conditions. Also, the migratory potential was significantly lower for control gMSCs when compared to ZE-gMSCs and significantly higher for ZE-gMSCs when compared to BF-gMSCs or BFL-gMSCs. Conclusions: The results of the present study highlight a possible risk of cytotoxicity when using resin based-materials in dental practice, but they cannot be d irectly extrapolated to *in vivo* situations.

KEYWORDS: *Composite resin, cytotoxicity, gingival stromal mesenchymal cells, functionality.*

Introduction

The introduction of composite resins in the field of dentistry over 50 years ago is one of the many successes of medical research [1].

Due to remarkable aesthetic and mechanical properties, composite resins have gradually gained prevalence over other dental materials, being currently the materials of choice for direct dental restorations [2].

Composite resins are complex materials, composed of a variety of monomers, filler particles, initiators, activators and other additives [3].

The filler particles introduced into the inorganic phase of composite resins provide their mechanical strength, their optical and radiopacity properties, and the organic monomers are mainly responsible for the viscosity, polymerization shrinkage and absorption of water, ensuring in the meantime the handling and shaping of the material [4,5].

Over time, the structure and the content of composite resins have been constantly modified, to obtain new materials with improved aesthetic properties and increased finishing/polishing capacity, providing in the meantime optimal mechanical strength [6,7] and stability in the oral environment [8,9].

Giomers are considered hybrid materials that incorporate the best properties of composite resins and glass ionomers, such as mechanical strength, esthetics and protection against carious lesions, by constantly releasing and recharging with fluoride [10,11].

The Giomer concept in based on PRG (Pre Reacted Glass) technology: a glass core surrounded by a glass ionomer phase enclosed within a polyacid matrix, containing fluoroaluminosilicate glass particles that have previously reacted with the polyacrylic acid [12].

Due to their fluoride release and recharge abilities obtained through PRG technology, giomers were introduced in the class of smart materials [12].

The morphological, functional and mechanical integrity of giomer restorations was

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comparable to that of resin composite restorations [12].

However, there are data in the literature sustaining that the long-term clinical success of giomers is relatively lower than that of composites [11].

Although the remarkable evolution of resin-based materials has led to a substantial improvement in their properties, these materials are still associated with some disadvantages that may compromise the clinical performance and longevity of dental restorations.

Polymerization shrinkage and the related stress are still important shortcomings of composite resins, which can induce microinfiltration and marginal discoloration, secondary caries, pulpal pathology and post-operative sensitivity [13,14].

Moreover, the release of toxic chemical compounds due to incomplete polymerization or progressive degradation is another major disadvantage of composite resins [5,15,16] which can induce systemic or local oral toxic effects [5,17,18,19].

Irritation of the oral mucosa, hypersensitivity reactions and lichenoid or anaphylactoid reactions have been described after the placement of resin composite restorations [20].

The placement of resin composites in the proximity of marginal periodontal tissues could be associated with cell-related negative events such as cell death, inflammation, apoptosis, reduced viability [21], mutagenic or genotoxic effects [22] as well as with a compromised healing of traumatized gingival tissues during restoration placement maneuvers [21].

The clinical consequences are gingival inflammation and loss of periodontal attachment [22].

In deep cavities, the non-released residual monomers may diffuse through dentinal tubules causing pulpal inflammation [19,23].

Residual monomers are released into the oral environment mostly during the first 24 hours after their clinical placement with a progressively decrease in release and toxicity after that [3,24].

The literature reported various methods for assessing the cytotoxicity of dental materials, such as tests for estimating the amount of ribonucleic acid and the level of damage to its chains or assessing the level of glutathione in cells or the severity of apoptotic action [25].

The most common method for the evaluation of the cytotoxicity of materials is based on cell cultures; the use of oral cells allows a more accurate experimental reproduction of oral cavity environment [25].

Among these latest methods, the MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) is simple, fast and accurate test [24,26] that provides important information on cellular metabolic activity and represents a good indicator of cell viability, proliferation and cytotoxicity [27].

This test appreciates the ability of mitochondrial enzymes of viable cells to catalyze the reduction reaction of soluble tetrazolium salt in insoluble, red formazan crystals, quantified through a spectrophotometric approach [25].

Thus, the more intense the color of the final product, the higher the number of metabolic active viable cells [25].

The present study aimed to evaluate and compare the cytotoxicity of some conventional resin composites on the progenitor periodontal pool represented by mesenchymal stromal cells of gingival origin (gMSCs) by performing functionality tests and the MTT assay.

We tested the null hypothesis, which considered that the cytotoxicity of tested materials did not differ.

Material and methods

The study workflow

The present study was carried out at the Discipline of Periodontology, Faculty of Dentistry, *Iuliu Haţieganu* University of Medicine and Pharmacy, Cluj-Napoca and the Discipline of Reproduction and Reproductive Pathology, University of Agriculture Sciences and Veterinary Medicine, Cluj-Napoca, after obtaining the agreement of the Ethical Bord of *Iuliu Haţieganu* University (No. 268 of 30.07.2019).

Gingival tissues were sampled from patients undergoing periodontal surgeries after signing the informed consent regarding the study.

The research adhered to the Declaration of Helsinki with respect to experimentation involving human subjects.

The development of the experiments using stem cells followed EU and national laws.

For this study, gMSCs previously isolated and fully characterized by our team [28] according to the standard criteria recommended by the International Society for Cell Therapy [29] were used.

Cells at passage 4 were put in contact with experimental materials and functionality and MTT assays were evaluated.

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Preparation of material samples

Three different restorative materials were used for the present study: Zenit® (President Dental, GERMANY), Beautifil II® LS and Beautifil II® (Shofu Dental Corporation, JAPAN). The main characteristics and composition of the tested materials are provided in Table 1.

Table 1. Main characteristics and composition of tested materials.

Type of material	Manufacturer	Composition and characteristics
Beautifil II® LS	Shofu Dental Corporation, JAPAN	Giomer (PRG technology) ►*Matrix*: Bis-GMA, TEGDMA, UDMA, Bis-MPEP ►*Filling*: S-PRG filler based on Aluminofluoro-borosilicate glass, mean particle size 0.4 µm ►*Filling content (weight%):* 83%
Beautifil II®	Shofu Dental Corporation, JAPAN	Giomer (PRG technology) ►*Matrix*: Bis-GMA, TEGDMA ►*Filling*: S-PRG filler based on Aluminofluoro-borosilicate glass, nano fillers (10~20nm) ►*Filling content (weight%): 83%*
Zenit	President Dental, GERMANY	Compozit nano-ceramic ►*Matrix*: UDMA, Bis-GMA, BDDMA ►*Filling*: glass filler 0,7 µm, pyrogenic silica 12 nm, nanoparticles 0,6 µm ►*Filling content (weight%): 83%*

S-PRG Technology=surface pre-reacted glass ionomer; Bis-GMA=bisphenol-A-diglycidyl methacrylate; TEGDMA=triethyleneglycol dimethacrylate; UDMA=urethane dimethacrylate; Bis-MPEPP=bisphenol A polyethoxy dimethacrylate; BDDMA=Butanediol dimethacrylate.

For each material, 15 disc-shaped samples were prepared by placing the material in a 6mm diameter, 1mm thick mold.

After removing the excess material, the samples were covered with a plastic foil and light-cured for 20 s using a light activation unit (Demi LED Curing Unit, Kerr Corporation, Orange, CA, USA).

Subsequently, the samples were sterilized by ethylene oxide gas for 2h at 56°C followed by degassing for 12h.

Preparation of the cell suspension and the placement of the material discs

The influence of components released from resin composites on gMSCs was evaluated by placing the material discs in 0.4µm hanging inserts (Millicell-® TM, Cell Culture Inserts, Merck KGaA, Darmstadt, Germany) placed afterwards into 24-well cell culture plates (CytoOne, Cell Culture, Mineapolis).

Control wells were free of resin composites.

Cells grown to 80% confluence were detached from culture plates using trypsin-EDTA (1:4) and counted with a hemocytometer (Isolab Laborgeräte GmbH, Wertheim Germany).

The adjustment of cell number was performed.

The cell-containing solution was seeded onto 24-well cell culture plates (CytoOne) and the discs-containing inserts were introduced.

Cells grown in relation with the tested resin-based materials were named based on each material code (ZE-gMSCs, BF-gMSCs, and BFL-gMSCs).

Functionality tests

Colony-forming unit-Fibroblast assay

A colony-forming unit-fibroblast (CFU-F) assay was performed to evaluate the behavior of gMSCs grown in association with resin-based material samples.

The gMSCs cultured in standard conditions without material discs were used as control samples. gMSCs were co-cultured with dental material discs in a complete propagation medium at 1cell/cm on 100cm^2 concentration and incubated at 37°C in a 60-90% humidified atmosphere enriched with 5% CO2.

Following a 14-day culture, cells were formalin-fixed, stained with Crystal Violet 0.5% Alcoholic Solution (Sigma-Aldrich, St. Louis) for 30 minutes and examined with a phase

contrast inverted light microscope (NikonTS100, Nikon Instruments, Europe).

Colonies containing more than 50 cells were counted and the CFU-F efficiency was calculated using the formula: CFU-F efficiency=(counted CFU-F/cells originally seeded) x 100 [29,30,31].

Migratory potential assay

A total of $2.5x10^2$ cells were seeded in a standard culture medium (1ml/well) with the hanging drop method.

Following a 48-h cultivation, the spheroid aggregates were harvested and transferred to 6-well plates (CytoOne, Cell Culture, Minneapolis) coated with 1% gelatin (Sigma-Aldrich, St. Louis) in PBS (Sigma-Aldrich, St. Louis) to facilitate the attachment of the aggregates and to increase the migratory potential of gMCSs.

Following a 7-day culture, the aggregate size and the gMSCs coverage area were measured (NIS-Elements D3.0 Laboratory Imaging software) and the area of migration was calculated using the formula: migration area=gMSCs migration area-aggregate size [30,31].

MTT assay

MTT assay was performed to evaluate the viability of gMCSs following 24h of co-cultivation with dental material discs.

A concentration of $8x10^5$gMSCs/mL cells was seeded onto a 24-well plate. Following a 24-h culture the discs were placed onto the monolayer and after 24h they were removed and 100µl MTT solution/well (Sigma-Aldrich, St. Louis, MO) was added.

Following a 4h-incubation at 37°C, the MTT solution was removed and 150µl DMSO (dimethyl sulfoxide) (Fluka, Buchs, Switzerland) was added to dissolve the formazan crystals.

To determine the cell viability, the absorbance density values were assessed at 450nm using a BioTek Synergy 2 reader (Winooski, VT).

Comparing to dead cells, only the viable cells were able to produce a dark blue formazan product.

The percentage of the viable cells was calculated using the formula: cells viability (%)=[(sample abs)/(control abs)] x100.

Statistical analysis

The statistical analysis was carried out using MedCalc® Statistical Software version 20.111 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2022) program.

A Shapiro-Wilk test was performed to verify the normal distribution of the variables.

Continuous variables were reported as mean±standard deviations.

The measured values for the tested materials were compared using the ANOVA test.

Whenever the ANOVA test indicated significant differences between the analyzed groups, a post-hoc analysis (Tukey's honest significant difference test) was carried out to identify specific differences among the studied groups.

Correlations between measurements were verified with Pearson correlation coefficient. A value of $p<0.05$ was considered statistically significant.

Results

Functionality tests

The CFU-F assay and the migratory potential of gMSCs were investigated to evaluate if the products released from the tested materials have a negative impact on the functions of gMSC.

Formation of colonies

The ability to form colonies was demonstrated for both gMCSs cultured in standard conditions and in relation with the resin-based material samples.

However, the ANOVA test identified significant differences regarding the number of colonies formed in association with different resin-based materials (p=0.007).

According to the post-hoc analysis, the frequency of colony-forming cells was significantly lower for the BF-gMSCs compared to gMSCs control group (p=0.012) and for the BFL-gMSCs samples compared to gMSCs control group (post-hoc *p*-value=0.014).

The frequency of colony-forming cells associated with ZE discs was lower compared to that of cells cultured under normal conditions (post-hoc *p*-value=0.510) and higher than the frequency of colony-forming cells associated with BF discs (post-hoc *p*-value=0.089) and BFL discs (post-hoc *p*-value=0.102), but without any statistical significance (Figure 1).

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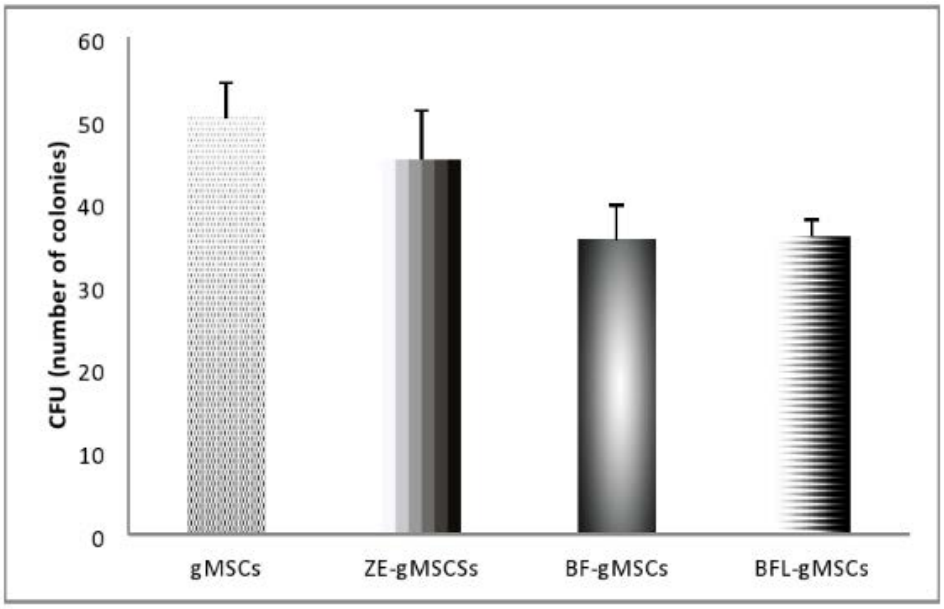

Figure 1. Graphical representation of the colony formation efficiency of gMSCs cultured under standard conditions (control=50.333±4.16333) and in association with restorative materials (ZE=45.333±5.859, BF=366±2). p values<0.05 for BF-gMSCs and BFL-gMSCs samples compared to the control samples. ZE=Zenit, BF=Beautiful II LS, BFL=Beautiful Flow Plus F03.

The migratory potential

In the present study, statistically significant differences between the migratory potential of gMSCs cultured under different conditions were identified by the ANOVA test (p=0.000). Thus, the post-hoc analysis revealed a significantly reduced migratory potential for control gMSCs when compared to ZE-gMSCs (post-hoc *p*-value=0.001). Also, ZE-gMSCs demonstrated a significantly higher migratory potential when compared to BF-gMSCs (post-hoc *p*-value=0.000) or BFL-gMSCs (post-hoc *p*-value=0.000) (Figure 2).

There were no statistically significant differences between the migratory potential of gMSCs cultured in standard conditions when compared to BF-gMSCs group (post-hoc *p*-value=0.692) or BFL-gMSCs group (post-hoc *p*-value=0.390).

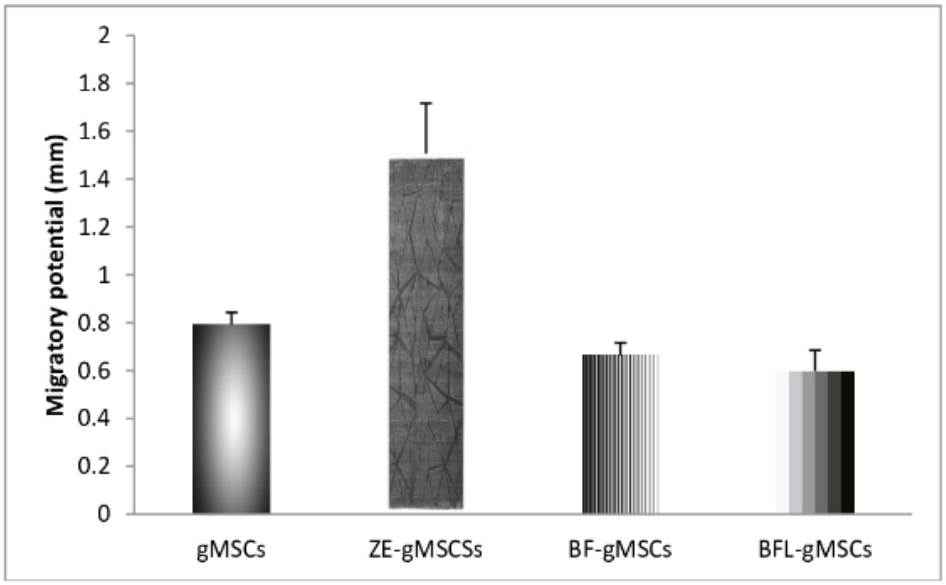

Figure 2. Graphical representation of mean migratory potential (mm) of gMSCs cultured under different conditions: control gMSCs (0.792±0.050), ZE-gMSCs (1.507±0.210), BF-gMSCs (0,665±0,051), BFL-gMSCs (0.5996667±0,0862226). p values<0.05: ZE-gMSCs compared with control gMSCs, ZE-gMSCs compared with BF-gMSCs, and ZE-gMSCs compared with BFL-gMSCs. ZE=Zenit, BF=Beautiful II LS, BFL=Beautiful Flow Plus F03.

MTT assay

The MTT assay was performed to evaluate the viability of gMSCs cultured in standard conditions or in association with the resin-based material samples, 24h after cultivation. No significant differences related to viability of gMSCs grown in different conditions were identified by the ANOVA test (p=0.551). However, the lowest viability scores were observed for ZE-gMSCs, while control gMSCs were associated with the highest cell viability score. Small, non-significant differences were identified when comparing cell viability values between ZE-gMSCs and BF-gMSCs groups (post-hoc *p*-value=0.985), ZE-gMSCs and BFL-gMSCs groups (post-hoc *p*-value=0.847) or BF-gMSCs and BFL-gMSCs groups (post-hoc *p*-value=0.964) (Figure 3).

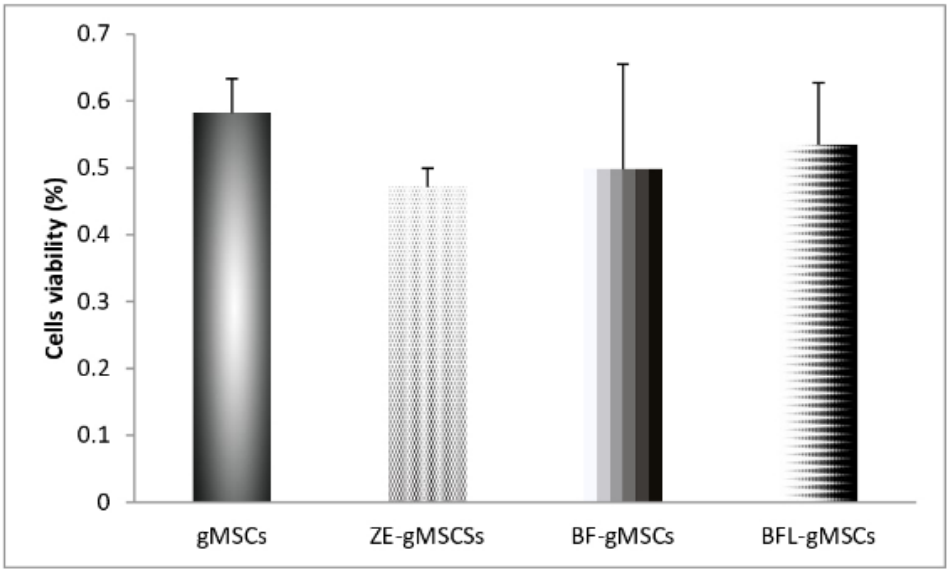

Figure 3. Graphical representation of the viability (%) evaluated by the MTT test at 24h: control-gMSCs (0.582±0.05), ZE-gMSCs (0.471±0.034), BF-gMSCs (0,498±0,156) and BFL-gMSCs (0.535±0,092). p values<0.05: ZE-gMSCs compared to the other groups ZE=Zenit, BF=Beautiful II LS, BFL=Beautiful Flow Plus F03.

MTT assay and functionality tests correlation assessment

For the experimental materials, Pearson correlation coefficient test revealed no correlation between the MTT assay and the migratory potential: ZE (r=-0.726, p=0.483), BF (r=-0.630, p=0.566), BFL (r=-0.944, p=0.214) or between the MTT assay and CFU-F test: ZE (r=0.233, p=0.850), BF (r=0.976, p=0.140), BFL (r=0.941, p=0.219). For gMSCs control group, a significant positive correlation was identified between the MTT assay and the migratory potential (r=1, p=0.019).

However, there was no correlation between the MTT assay and CFU-F test (r=0.287, p=0.815) (Table 2).

Table 2. Correlation between MTT assay and functionality tests.

Sample		Migratory potential **N=3**		CFU-F test **N=3**	
		r-value	*p-value*	*r-value*	*p-value*
ZE-gMSCs	MTT assay **N=3**	-.726	.483	.233	.850
BF-gMSCs		-.630	.566	.976	.140
BFL-gMSCs		-.944	.214	.941	.219
gMSCs		1.000*	.019	.287	.815
*Correlation is significant at *p*-value<0.05					

Discussions

The present study investigated the cytotoxiciy of some commercial dental materials on MSCs isolated from gingival tissues, since these materials are not inert, and no ideal product has been recommended yet. The cytotoxicity of the tested materials was directly assessed by the MTT assay to evaluate cell viability and indirectly through functionality tests (CFU-F and migratory potential).

In general, dental materials used to replace damaged or lost dental tissues should be chemically stable and inert to oral cavity tissues [32].

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However, according to the literature, all dental materials can have negative local effects [32].

The biocompatibility of dental materials and their effects on the oral cells have significant clinical importance, regarding their continuous evolution on the market and the lack of information concerning their potential for cytotoxicity. Placed in close contact with gingival tissues, resin-based materials can induce some harmful effects on oral cells, due to local release of leached compounds as a result of incomplete polymerization, degradation processes or erosion [21,33,34].

As other previous reports [17,35,36], the present research chose MSCs originating from gingival tissue because human cell lines are considered more sensitive to cytotoxicity tests in comparison with animal cell lines [37].

Moreover, the use of gMSCs seemed a logical consequence of the proximity of resin composite restorations to gingival tissues in many clinical circumstances [28].

Also, gMSCs are abundantly available due to the facile accessing of gingival tissues [28,38].

For the MTT assay, the most commonly used cells are gingival fibroblasts and keratinocytes isolated from the oral epithelium [24,25].

The present study evaluated cell viability by using the MTT assay due to related advantages associated with this approach such as simplicity of execution, accuracy, rapidity and reliability as well as increased sensitivity [28,39].

The MTT assay is a good indicator of cell viability, proliferation and cytotoxicity [27,39].

Our study did not identify statistically significant differences regarding the viability of gMSCs cultured in standard conditions compared to those cultured in association with the resin-based material samples.

However, the viability of gMSCs cultured in the absence of restorative materials was increased compared to that of gMSCs cultured in association with composite discs after 24h.

In addition, the lowest cell viability score was recorded for the ZE-gMSCs sample. Thus, the giomers (Beautifil II® LS and Beautifil Flow Plus® F03) had a lower effect on cell viability compared to the conventional composite.

There is little research in the literature regarding the cytotoxicity of giomers compared to that of other restorative dental materials. The reduced effects of the giomers on cell viability highlighted by this study are in agreement with several earlier reports. Koohpeima et al. evaluated the cytotoxic effects of nanohybrid composites, giomers, conventional, resin-modified and silver-reinforced glassionomers on gingival fibroblasts using the MTT assay. The study revealed significantly increased cytotoxicity for silver-reinforced glassionomers in association with several cell concentrations and for nanohybrid composites in association with all cell concentrations. The research did not report a significant cytotoxic effect for giomers, conventional or resin-modified glassionomers on gingival fibroblasts. The reduced cytotoxicity of giomers has been attributed to their surface structure which greatly influences the biocompatibility of the material and the lower amount of released toxic compounds [40].

Similarly, the research conducted by Tamilselvam et al. revealed a significantly reduced cytotoxicity of the giomers on gingival fibroblasts, compared to conventional and reinforced glassionomers [26].

In the present study, gMSCs cultured in association with BF and BFL discs had a significantly lower CFU-F capacity compared to gMSCs cultured under normal condition. Thus, the giomers had a significant more negative impact on the ability of gMSCs to form colony-forming units, according to the CFU-F assay. The migratory potential of gMSCs cultured in association with ZE discs was significantly increased compared to that of gMSCs cultured in normal conditions or in association with the giomers.

Therefore, depending upon the performed functionality test, the present study identified significantly reduced-functionality of gMSCs cultured in association with BF and B discs, compared to that of gMSCs cultured in normal conditions or in association with ZE discs.

The results of the correlation assessment between MTT assay and functionality tests (migratory potential and CFU-F) for each experimental material was not significant. The lack of correlation could be explained by the fact that each test evaluates differently the cells behaviour.

The significant differences highlighted in our study upon cells functionality in association with the tested materials can be explained by the different organic structure of each material.

According to the literature, Bis-GMA, UDMA, TEGDMA and HEMA monomers released from the resin matrix in the oral environment are mainly responsible for the cytotoxicity of resin-based materials [5,19,41].

Recent data suggests that hydrophilic monomers like HEMA or TEGDMA are released

in higher amounts compared to Bis-GMA or UDMA [5,42].

The harmful effects of Bis-GMA and TEGDMA monomers was reported on multiple cell lines, such as human gingival cells, pulpal fibroblasts, monocytes or erythrocytes [19].

While Bis-GMA is present in the organic matrix of all tested materials, TEGDMA monomer is only present in the giomers. TEGDMA is a dental co-monomer introduced in the organic phase of resin-based materials to obtain appropriate viscosities [43].

As reported by the literature, TEGDMA can be released from resin composites in significant quantities [44].

Due to its low molecular weight and high hydrophilicity, TEGDMA monomer can penetrate in all biological compartments such as nuclei, being capable to influence physiological processes such as cell growth and differentiation [44,45,46].

Furthermore, in a time and concentration-dependent manner, TEGDMA can also generate cellular stress through formation of reactive oxygen species causing apoptosis or necrosis [46,47].

Conclusion

The results of the present study have an important contribution in restorative dentistry field and should be taken into account when placing resin-based materials in the proximity of periodontal tissue.

However, they cannot be directly extrapolated to *in vivo* situations, so further research with conclusive evidence should be conducted for a better understanding the possible risk of using resin based-materials in dental practice.

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Conflict of interests

None to declare.

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Corresponding Author: Alexandra Roman, Department of Periodontology, „Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, e-mail: veve_alexandra@yahoo.com