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RESEARCH AND EDUCATION

Effects of adding graphene fibers to polymethyl methacrylate on biocompatibility and surface characterization

Ildefonso Serrano-Belmonte, DDS, PhD,[a] Francisco Javier Cascales-Pérez, DDS,[b] Virginia Pérez-Fernández, DDS, PhD,[c] Ascensión Martínez-Cánovas, DDS, PhD,[d] María Rosario Tudela-Mulero, BS, PhD,[e] and Juan Ignacio Rosales-Leal, DDS, PhD[f]

ABSTRACT

Statement of problem. Interim fixed prostheses are used provisionally to provide esthetics and maintain function until placement of the definitive prosthesis. Polymethyl methacrylate (PMMA) has been widely used as an interim material but has mechanical limitations that can be improved with the addition of nanomaterials such as graphene fibers (PMMA-G). However, studies on the biocompatibility of this material are lacking.

Purpose. The purpose of this in vitro study was to determine the biocompatibility and cytotoxic effects of PMMA compared with PMMA-G in periodontal ligament stem cells (PDLSCs) by measuring the viability and cell apoptosis of those cells subjected to different concentrations of both compounds by elution, as well as the surface characterization of these materials.

Material and methods. Sterile Ø20×15-mm specimens of PMMA and PMMA-G were covered with Dulbecco modified Eagle medium for 24 hours to be the subsequent eluent. PDLSCs were seeded in 6 plates of 96 wells at dilutions 1/1, 1/2, 1/4, and 1/8 for each material. Three plates for the cell viability assay with MTT and 3 plates for the cell apoptosis assay with Hoechst 33342 staining were used in turn to subdivide the measurements at 24, 48, and 72 hours. The Kruskal-Wallis test was used to compare the data obtained in the different dilutions at different times and the Mann-Whitney test to compare both materials. Topography and wetting were analyzed for surface characterization. The Student *t* test of paired measurements was used to compare the different surfaces for each parameter (α=.05 for all tests).

Results. In both the cell viability assay (MTT) and the cell apoptosis assay, the test did not identify statistically significant differences in PMMA and PMMA-G with respect to the control group in the different dilutions at different times ($P>.05$). When comparing both materials, no statistically significant differences ($P=.268$) were found in either trial. PMMA-G had lower roughness and kurtosis and higher wetting than PMMA.

Conclusions. Both PMMA and PMMA-G were found to be biocompatible materials with no significant differences between them after cell viability and apoptosis testing. PMMA-G had higher wettability and lower roughness than PMMA. (J Prosthet Dent 2025;133:281.e1-e8)

Commonly used materials for interim prostheses include composite resins and acrylic resins,[1–5] especially polymethyl methacrylate (PMMA).[6] Different nanoparticles, including graphene, in the form of tubes, sheets, fibers, or spheres, have been added to PMMA to improve properties.[7,8] Graphene is a 2-dimensional nanomaterial consisting of a single layer of carbon atoms arranged in a honeycomb lattice. Its hardness, strength, flexibility, transparency, biocompatibility, and high thermal and electrical conductivity have made the use of graphene common in many fields.[9] Graphene has been manufactured in sheets and, through various physical and chemical modifications, derivatives such as graphene oxide (GO) and reduced graphene oxide (rGO) have been obtained.[10]

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[a]Assistant Professor, School of Dentistry, Faculty of Medicine, University Dental Clinic, Morales Meseguer General University Hospital, University of Murcia, Murcia, Spain.
[b]Graduate student, School of Dentistry, Faculty of Medicine, University Dental Clinic, Morales Meseguer General University Hospital, University of Murcia, Murcia, Spain.
[c]Professor, Institute of Biomedical Research (IMIB-Arrixaca), University of Murcia, Murcia, Spain.
[d]Assistant Professor, School of Dentistry, Faculty of Medicine, University Dental Clinic, Morales Meseguer General University Hospital, University of Murcia, Murcia, Spain.
[e]Researcher, Scientific and Technical Research Area (ACTI), University of Murcia, Spain.
[f]Professor, Faculty of Dentistry, University of Granada, Granada, Spain.

Clinical Implications

The addition of graphene fibers to polymethyl methacrylate led to a material that was biocompatible with periodontal ligament stem cells. In addition, the graphene coating reduced roughness and increased wettability.

Recently, nanoreinforced biopolymer disks with functionalized graphene fibers have appeared for milling by computer-aided design and computer-aided manufacturing (CAD-CAM) (G-CAM), which, because of improved physical properties have been suggested as a material of definitive use.[7–9] The incorporation of graphene fibers in PMMA resins to improve their properties may change the biocompatibility of the material.[11] Analyzing the biological effects that these materials can cause during their clinical application with biocompatibility and cytotoxicity tests is necessary.[12] The cytotoxicity tests allow structural changes in the cell, alterations in cell proliferation, or alterations in any of its functions to be measured.[13,14] Periodontal ligament stem cells (PDLSCs) are found with a wide potential for differentiation in vitro, making them suitable for evaluating dental materials in vitro, and are biocompatible with oral tissues.[15–23]

The interactions of solid surfaces with tissue cells are greatly influenced by the physical properties of the surface.[24] The wettability and topography of a polymer surface plays a critical role in cell surface interaction and behavior. Therefore, understanding how these physical parameters control the surface adhesion of fibroblast cells is of significance in the design of biomaterial surfaces. Surface energy influences the contact area of the cell membrane with the substrate, while the profile of the membrane could change depending on the wettability of the adjacent solid. Cell adhesion is then mediated by wetting properties.[25–31]

The main objective of this study was to determine the biocompatibility and cytotoxic effects of PMMA compared with PMMA-G in PDLSCs by measuring the viability, proliferation, and apoptosis or cell death of PDLSCs subjected to different concentrations of PMMA and PMMA-G by elution. Another objective of this research was to characterize the surface by topographic and wetting analysis. The null hypothesis was that no significant differences would be found in the cell viability, number of apoptotic or dead cells, wettability, and roughness of PMMA and PMMA-G.

MATERIAL AND METHODS

Human dental pulp and periodontal ligament were obtained from impacted third molars from 14 healthy volunteers who provided written informed consent according to the guidelines of the Ethics Committee of the University of Murcia. To generate single-cell suspensions, the pulp and periodontal ligament was gently removed and immersed in a solution of 3 mg/mL collagenase type I (Sigma-Aldrich) for 1 hour at 37 ºC. The cells obtained after this treatment were seeded into two 25-cm^2 plastic tissue culture flasks (BD Biosciences) and incubated at 37 °C in a humid atmosphere containing 5% CO_2 for 3 days. On the third day, red blood cells and other nonadherent cells were removed, and fresh medium was added to allow further growth. The adherent cells were grown to 80% confluency and were defined as passage zero (P0) cells. To confirm the mesenchymal phenotype of the cells, the expression of different mesenchymal markers was studied. PDLSCs were chilled in liquid nitrogen at −196 ºC in the cell culture service of the University of Murcia. After warming, they were grown in 2 culture bottles of 75 cm^2 and 1 of 25 cm^2 and incubated at 37 ºC, 7.5% CO_2, and 85% relative humidity. To obtain sufficient cells to carry out the research, a total of 3 subcultures were performed, with a change of medium 4 days after each subculture.

After the favorable report of the study by the Biosafety Committee in Experimentation of the University of Murcia, the preparation of the extracts of both materials was carried out following the standard protocol established by the International Organization for Standardization (ISO) 10993–12 standard for preparation of specimens and reference materials. A Ø98.5×20-mm disk of PMMA (lot number L20021210002) and another of PMMA-G (G-CAM lot number L18121120072) with the same dimensions were provided by Graphenano Dental SL. The disks were milled by CAD-CAM with a 5-axis dental milling machine (Ceramill Motion 2; Amann Girrbach AG) to obtain Ø20×15-mm cylinders after the creation of a standard tessellation language (STL) file with these measurements. The specimens were sterilized in a class N autoclave at 134 ºC and 210 kPa for 30 minutes in sterilization bags. Sterile PMMA and PMMA-G specimens were coated with Dulbecco modified Eagle medium with 4.5 g/L glucose and phenol red supplemented with 10% fetal bovine serum (SBF), 4 mM glutamine, and 1% penicillin/streptomycin at a ratio of 0.2 g of material per mL of culture medium according to the ISO 10993–5 standard. As the PMMA cylinder weighed 4.26 g, it was immersed in 21.3 mL of culture medium, while the PMMA-G cylinder weighing 4.27 g required 21.35 mL. The specimens were left 24 hours in the CO_2 incubator at 7.5%, at 37 ºC, and humidity of 85%.

To obtain sufficient results to analyze, the cells were seeded in 6 plates of 96 wells with a seeding density of

3000 cells/well and incubated at 37 °C, 7.5% CO_2, and 85% humidity for 24 hours. Subsequently, from these 6 plates, 3 plates were separated to be used with 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) and 3 plates for staining with Hoechst to, in turn, subdivide them for measurements at 24, 48, and 72 hours. In each of the plates, eluent at dilutions 1/1, 1/2, 1/4, and 1/8 for each material were used. The medium was removed from each of the wells, so that each dilution could be applied in a column with 6 rows of wells. In addition to dilutions, 12 controls and 2 blank and water controls were used. For cell culture and plate seeding, the essay was developed under sterile conditions in a type II biological safety cabinet with a high efficiency particulate air (HEPA) filter.

Cell proliferation was measured using MTT viability assay.[16] After culturing the cells and after the incubation time (24, 48, or 72 hours), Dulbecco modified Eagle medium with phenol red was replaced by 200 µL of Dulbecco modified Eagle medium without phenol red. The MTT reagent was added to each of the wells to remain at a final concentration of 1 mg/mL and incubated at 37 °C, 7.5% CO_2, and 85% relative humidity for 90 minutes. After incubation, the contents of the wells were removed, and 100 µL of dimethyl sulfoxide (DMSO) per well was added. The plate was shaken in an orbital stirrer to solubilize formazane for 5 minutes at 200 rpm. Finally, the absorbance was measured in a plate reader (FLUOstar Omega; BMG LABTECH) at a wavelength between 520 and 580 nm (Fig. 1). In this case, it was measured at 550 nm, and a reading was also simultaneously made at 690 nm to subtract the background.

To assess cell apoptosis morphologically, Hoechst 33342, which emits a blue fluorescence when excited by ultraviolet light, was used. This compound strongly marks the cell nuclei of altered cells.[17] After incubation (24, 48, and 72 hours), the medium was removed, and cells were fixed with the Casnoy fixative solution (ratio 3/1 absolute methanol and glacial acetic acid). The plates were allowed to dry for 24 hours and then were then incubated for 15 minutes in darkness and at room temperature with a Hoechst 33342 solution in phosphate-buffered saline (PBS) with a concentration of 1 mg/mL. After removing the solution and adding PBS, images were made with an inverted microscope (Eclipse TE2000-U; Nikon) of each of the concentrations of the 3 plates (24, 48, and 72 hours) at 50 µm scale. The images were processed in a software program (Image J, version 1.53q; National Institutes of Health), the FIJI macro developed by the image service of the University of Murcia and the "Cell Counter" plugging developed by Kurt de Vos. Living cells showed normal morphology and low blue fluorescence, while dead cells exhibited altered morphology and intense blue fluorescence. In this way, the pyknotic and apoptotic nuclei were easily identifiable (Fig. 1).

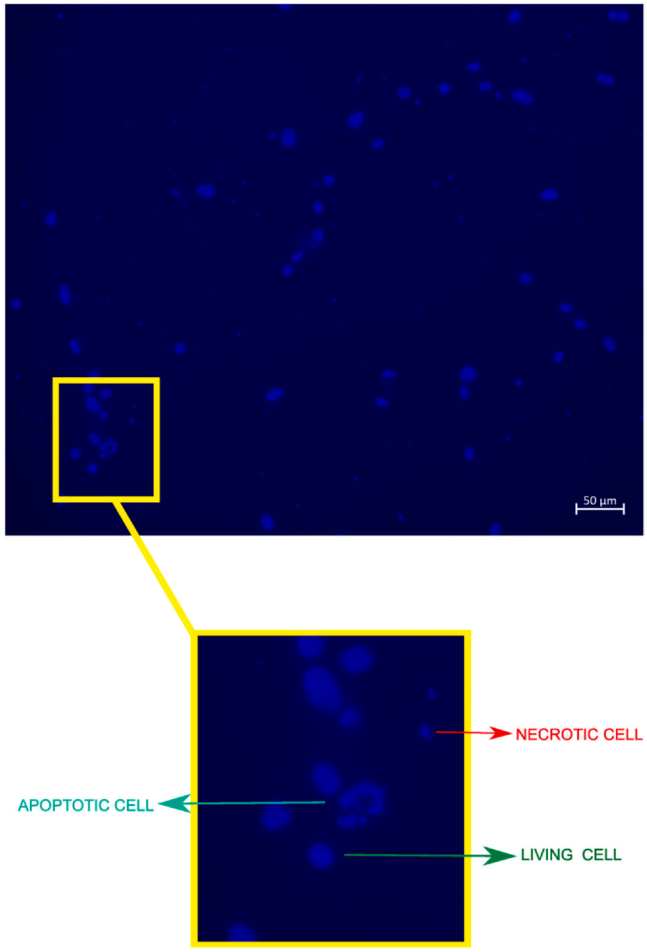

Figure 1. Inverted microscope image. (1) Apoptotic cell. (2) Necrotic cell. (3) Living cell.

Topographies were acquired with a white light confocal microscope (PLµ; Sensofar-Tech), examining 3 disks per group and acquiring 3 topographies per disk with an EPI ×50 objective (scan size 292×214 µm). The microscope software program provided data on the topographic parameters: arithmetic mean roughness (Sa), maximum relative height (Sp), maximum relative depth (Sv), Sp+Sv (St), root mean square roughness (Sq); skewness (Ssk), and kurtosis (Sku).

Wettability was evaluated on 3 disks from each group by measuring the contact angle using the axisymmetric drop shape analysis-contact diameter (ADSA-CD) technique. The contact angle (θ) was determined from a 1-mL sessile drop deposited on the surface with a micropipette and purified water (Milli-Q; Merck KGaA) for the measurements. Measurements were made in triplicate for each disk.

The data were descriptively analyzed using bar graphs and a boxplot according to which 2 materials and

control or only 2 materials are summarized. The topographic analysis data were summarized using mean and standard deviation. The data had been analyzed previously with the Kolmogorov-Smirnov test to determine whether they were parametric or not. For the analysis of cell viability and for the study of apoptosis or cell death, the Kruskal-Wallis test was used to compare the data obtained in the different dilutions at different times, and the Mann-Whitney test to compare both materials. Regarding the topographic study, the Student *t* test of paired measurements was used to compare the different surfaces for each parameter (α=.05 for all tests).

RESULTS

The cells were exposed at 24, 48, and 72 hours and at dilutions of 1/1, 1/2, 1/4, and 1/8 and were then analyzed. In the cell viability test, absorbance was found to increase in both materials and in the control as the exposure time increased because the cells continued to maintain their metabolic activity and continued to reproduce exponentially, even when in contact with PMMA and PMMA-G (Fig. 2). From the Kruskal-Wallis test, no statistically significant difference was found between the PMMA or the PMMA-G groups and the control group when comparing all dilutions and different times (*P*=.126). PMMA and PMMA-G lack cytotoxic potential, since, as specified by the ISO 10993–5 standard, viability would have to be reduced by 70% with respect to the target for the material to be considered cytotoxic. When comparing the 2 materials, no statistically significant difference was found in cytotoxicity between them at the different dilutions: concentration 1/1 (*P*=.827), concentration 1/2 (*P*=.824), concentration 1/4 (*P*=.831), and concentration 1/8 (*P*=.513) (Fig. 3). For the apoptosis or cell death assay, a count of at least 200 cells of each concentration was performed and subjected to the Kruskal-Wallis test, with no statistically significant difference in the number of dead cells in PMMA and PMMA-G with respect to the control group with different dilutions. (*P*=.147). When

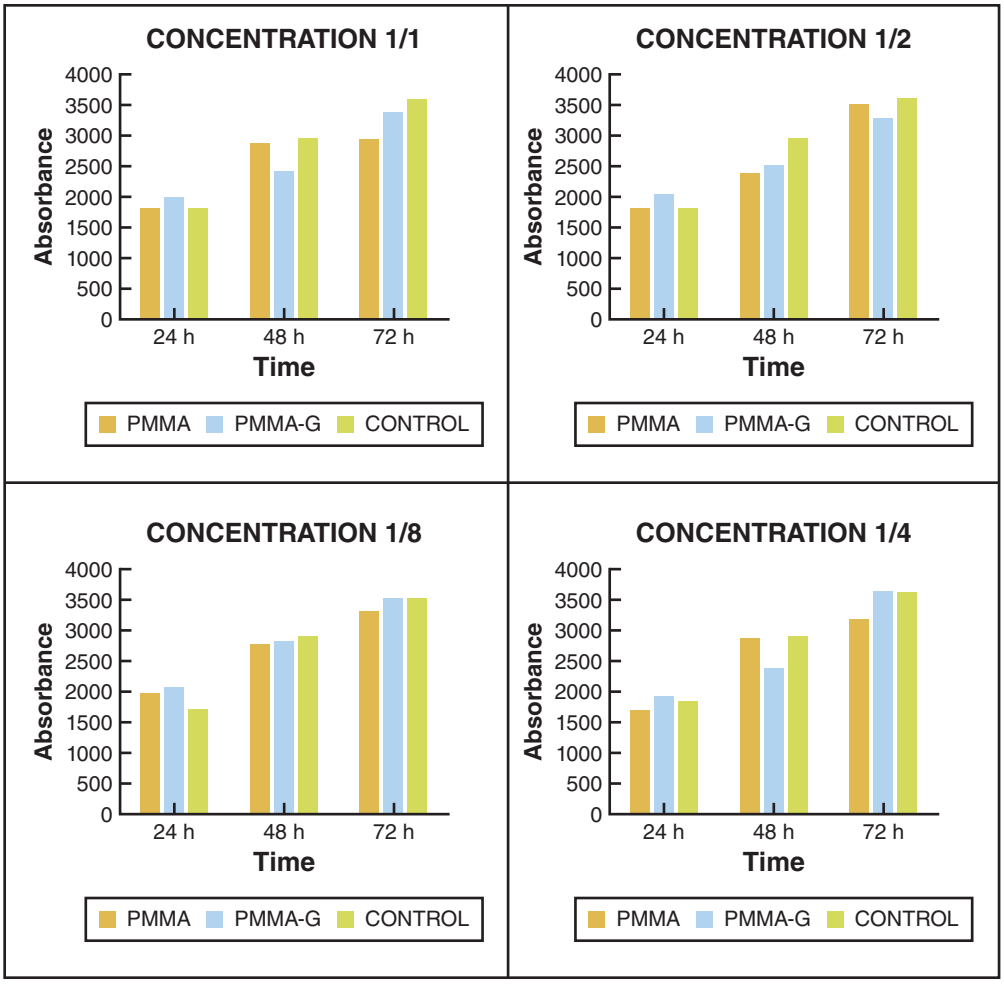

Figure 2. Absorbance and exposure time.

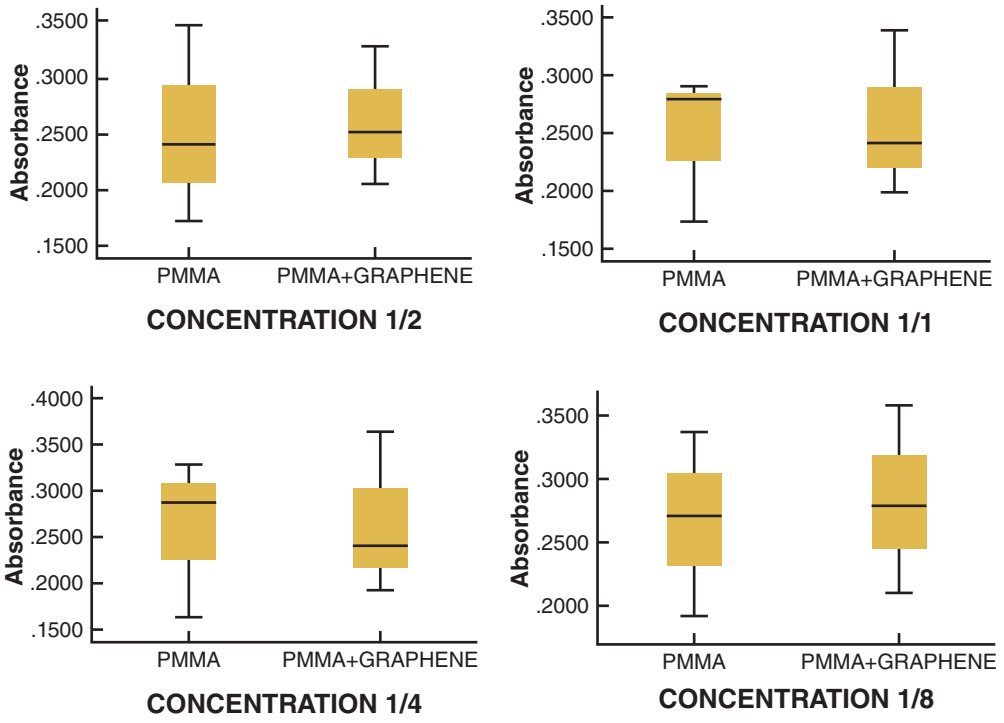

Figure 3. Mann-Whitney test and concentration.

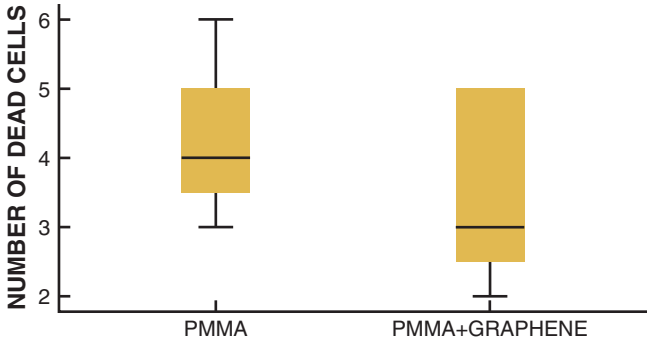

Figure 4. Mann-Whitney test and dead cells.

Table 1. Mann-Whitney test and dead cells

Dead-cells	PMMA	PMMA-G	*P*
Mean ±standard deviation	4.170 ±0.937	3.580 ±1.311	
Median (RI)	4 (3.50, 5.00)	3.58 (2.50, 5.00)	.268

comparing the 2 materials with the Mann-Whitney test, no statistically significant difference in the number of dead cells was found (P=.268) (Fig. 4, Table 1).

Table 2 shows roughness and wetting data. A significant influence of graphene coating was found in the roughness parameters and contact angle (P=.04). Roughness values were lower on grapheme-coated surfaces. Only skewness obtained similar values on both surfaces (P=.200). Wetting was higher (lower contact angle) in graphene-coated surfaces. Figures 5 and 6 show topographies of the noncoated and graphene-coated surfaces, showing different morphologies.

DISCUSSION

The use of graphene has generated contradictory results in the literature regarding its biocompatibility.[7–11,18,19] As

Table 2. Roughness and wetting data (mean ±standard deviation)

Parameter	PMMA	PMMA+GRAPHENE	*P*
Arithmetic average heigh (Ra)	1.6 ±0.2	0.73 ±0.03	<.001
Root mean square roughness (Rq)	2.1 ±0.3	0.87 ±0.03	<.001
Maximum height of peaks (Rp)	18 ±4	5 ±5	<.001
Maximum depth of valleys (Rv)	−17 ±−5	−3 ±1	<.001
Maximum height of the profile (Rt)	34 ±8	8 ±5	<.001
Skewness (Rsk)	0.4 ±0.8	0.08 ±0.07	.2
Kurtosis (Rku)	9 ±5	2.6 ±0.7	.002
Contact angle (degree)	67 ±9	61 ±7	.04

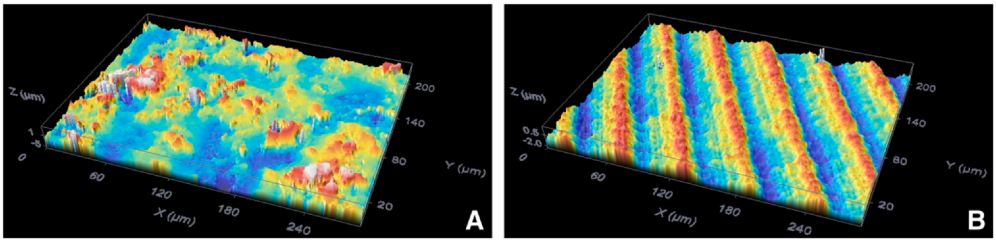

Figure 5. White light microscope micrographs (scan size 292×214 µm). A, Without graphene coating. B, With graphene coating.

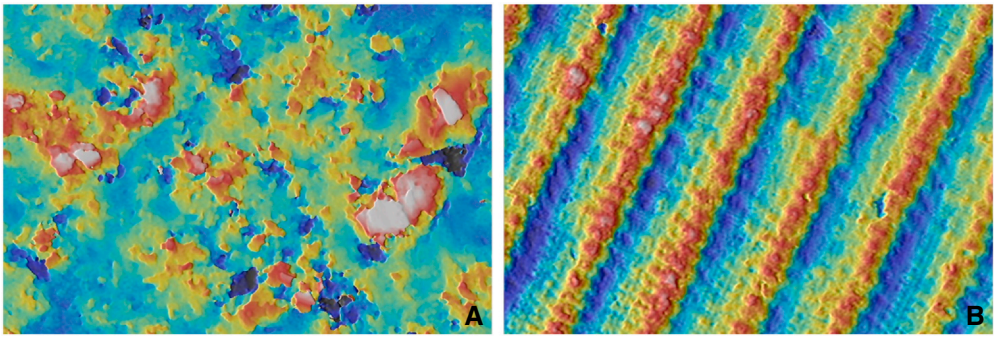

Figure 6. White light microscope micrographs. A, Without graphene coating. B, With graphene coating. (scan size 294×214 µm).

summarized by Liao et al,[18] these results may be derived from the structural diversity, quality, and different degrees of purity of oxidized graphene (GO), since the presence of impurities can have adverse effects on mammalian cells by inducing membrane damage and GO can improve cell viability or cause cell death depending on particle size, exposure time, and surface. Therefore, GOs with different levels of oxidation and impurities would interact differently when exposed to the same cell line. In addition, the oxygenated groups present in graphene oxide are several times higher than those found in functionalized graphene fibers,[7,11] the object of this study. After evaluating the present results with an MTT assay and the apoptosis or cell death assay in culture with PDLSCs, no significant differences in the biocompatibility of PMMA and PMMA-G were found, either in their different elutions or at different hours 24, 48, and 72. Therefore, the null hypothesis that no significant differences would be found in the cell viability or the number of apoptotic or dead cells was not rejected. These results were consistent with studies on GO such as that of Tahriri et al,[19] who reported a reduction in cytotoxicity in some cell types, including mesenchymal stem cells and human fibroblasts. García-Contreras et al[20] investigated the cytotoxicity of GO on cell proliferation in gingival fibroblasts, dental pulp cells, and human osteoblasts in culture and reported that GO had good compatibility and can contribute to cell proliferation. Incorporation of GO in PMMA was found in the present study to improve the physical, mechanical, chemical, and biological properties compared with PMMA without GO. Rodríguez-Lozano et al,[17] in a study on PDLSCs, stated that GO is a biocompatible compound that favors cell proliferation. However, and due to the causes mentioned, not all studies reported such favorable results on the properties of GO. Hashemi et al,[11] after performing tests on viability and genotoxicity in embryonic fibroblasts, reported that GO produced alterations in the cell cycle and induced cell apoptosis. Olteanu et al reported that, at high concentrations (40 mg/mL), GO reduced cell viability and altered the potential of certain cell organelles in the stem cells of human dental follicles. However, GO in low concentrations (4 mg/mL) has been reported to have a good safety profile, along with a high antioxidant defense.[22] In the present assay, the MTT test, a reliable and widely used method, was used to assess cell viability. However, the test has a drawback since the reagent used to provide the color continues to be introduced into the cells during the early stages of apoptosis, providing a false positive viability of cells that have already entered this state. Therefore, this study has implemented the apoptosis or cell death assay with Hoechst 33342, which allowed evaluation of the number of cells with apoptotic nuclei and ruled out the possible positive viability bias of MTT.[16,23]

Limitations of the study included that the thickness of the material immersed in the eluent (about 20 mm) was not representative of an interim prosthesis (close to 1 mm). Therefore, the ISO 10993–12 standard for irregular solid products was used.[14]

Regarding surface characterization, cellular adhesion was strongly dependent on wettability.[25,26] Wetting was

also related to the hydrophilic properties of a surface, with higher wetting on hydrophilic surfaces. Fibroblast adhesion is higher on hydrophilic than on hydrophobic substrates.[24] In the present study, the surface was found to be more hydrophilic after graphene coating, promoting wetting and higher fibroblast adhesion. The cell attachment was further enhanced and facilitated by a proper form and size of surface topography.[25] Surface roughness can affect cell adhesion at an early stage. It has been demonstrated that cell initial adhesion force and Ra are negatively correlated, and fibroblasts have greater attachment to smooth surfaces.[27] On rough surfaces, the actin cytoskeleton and fibronectin are disorganized, resulting in a low adhesion area.[28] In the present study, graphene-coated surfaces reduced roughness and increased cell adhesion. It has been demonstrated that human gingival fibroblast spread more widely and the formation of filopodia is increased on smooth surfaces with an Ra value <0.8 μm than on the rougher surfaces with an Ra value of 1.6 μm. The behavior of fibroblasts on rough compared with smooth surfaces has been described in several studies on titanium.[27,28] Osteoblast adhesion has also been reported to be reduced on highly rough surfaces and increased on less rough ones.[20] The current study confirmed this behavior on the 2 tested surfaces. A surface roughness with an Ra value between 0.15 and 0.25 μm has been proposed to provide optimal conditions for the growth of human gingival fibroblasts.[29] In this study, Ra values obtained on the graphene-covered surface were similar or closer to this value (0.7 μm) than the other material. In addition, the graphene coating leads to a kurtosis close to 3 compared with the uncoated surface, which obtains higher values. Kurtosis values above 3 indicate sharp peaks (leptokurtic distribution), whereas values below 3 indicate more rounded peaks with wider shoulders (platykurtic distribution).[30] In other studies,[29,30] it was concluded that cells prefer smooth leptokurtic surfaces to Gaussian or platykurtic surfaces to achieve maximum adhesion. These results would reject the null hypothesis of this study regarding surface characteristics. All these types of studies open the way to new research that can range from assessing the antimicrobial activity of GO on PMMA to the possible changes in the physical and mechanical properties that this union can exert, as they did in the study by Paz et al,[31] where they seemed to obtain improvements in the results of the parameters of the specimens with GO.

CONCLUSIONS

Based on the findings of this in vitro study, the following conclusions were drawn:

Both PMMA and PMMA-G had biocompatibility with PDLCs cells, allowing their viability and proliferation without significant differences between the materials.

No significant changes are observed in apoptosis or cell death by concentrations or by time of exposure.

The tested materials had different surface properties.

The graphene coating reduced roughness and increased wettability.

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Corresponding author:
Prof Ildefonso Serrano-Belmonte
School of Dentistry
Faculty of Medicine
University Dental Clinic
2nd Floor, Morales Meseguer General University Hospital
University of Murcia
Avenida Marqués de los Vélez s/n
Murcia 30008
SPAIN
Email: iserrano@um.es

CRediT authorship contribution statement

Ildefonso Serrano-Belmonte: Conceptualization, Methodology, Supervision; Validation. **Francisco Javier Cascales-Pérez**: Data curation, Writing- original draft preparation, Investigation. **Virginia Pérez-Fernández**: Writing- reviewing and editing, Software, Formal analysis. **Ascensión Martínez-Cánovas:** Writing-reviewing and editing, Validation. **María Rosario Tudela-Mulero:** Writing-reviewing and editing. **Juan Ignacio Rosales-Leal:** Writing- reviewing and editing, Data curation, Formal analysis.

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