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Article

Biological and physico-mechanical properties of poly(methyl methacrylate) enriched with graphene oxide as a potential biomaterial.

Propiedades físicas-mecánicas-biológicas del poli (metilmetacrilato) enriquecido con óxido de grafeno como potencial biomaterial.

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Abstract: Objective: To determine the cytotoxicity and effects of graphene oxide (GO) on cellular proliferation of gingival-fibroblasts, pulp-dental cells and human osteoblasts in culture, and to determine the physical, mechanical and biological properties of poly (methyl methacrylate) (PMMA) enriched with GO. **Material and Methods:** The GO was characterized with SEM. Cytotoxicity and cell proliferation were determined by the MTT bioassay. The physical-mechanical tests (flexural strength and elastic modulus) were carried out with a universal testing machine. Sorption and solubility were determined by weighing before and after drying and immersion in water. Porosity was evaluated by visual inspection. Data were analyzed with Student's t-test and Tukey's post-hoc ANOVA. **Results:** The GO has a heterogeneous morphology and a particle size of 66.67±64.76 µm. GO has a slight to no-cytotoxicity (>50-75% viability) at 1-30 days, and at 24 hours incubation of PMMA with GO significantly stimulates osteoblasts (45±8%, $p<0.01$). The physical and mechanical properties of PMMA with GO increase considerably without altering sorption, solubility and porosity. **Conclusion:** GO alone or with PMMA has an acceptable biocompatibility, could contribute to cell proliferation, cell regeneration and improve the physical-mechanical properties of PMMA.

Keywords: *polymethyl methacrylate; graphene oxide; biocompatible materials; mechanical tests; biological assay; cell proliferation*

Resumen: Objetivo: Determinar la citotoxicidad, proliferación celular del óxido de grafeno (OG) en cultivo con fibroblastos-gingivales, células-pulpares-dentales y osteoblastos-humanos, y las propiedades físicas, mecánicas y biológicas del polimetilmetacrilato (PMMA) enriquecido con OG. **Material y Métodos:** El OG fue caracterizado con SEM. La citotoxicidad y proliferación celular fueron determinadas con el bioensayo de MTT. Las pruebas físicas-mecánicas (Resistencia a la flexión y módulo elástico) se realizaron en una máquina de ensayos universales. La sorción y solubilidad se determinó por el pesaje antes y después de someterlas a desecamiento e inmersión en agua. La porosidad se evaluó por inspección visual. Los datos fueron analizados con pruebas t de Student y ANOVA post-hoc de Tukey. **Resultados:** El OG tiene una morfología heterogénea y un tamaño de partícula de 66.67±64.76 µm. El OG

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solo tiene de ligera a nula citotoxicidad (>50-75% de viabilidad celular) a 1-30 días. El PMMA enriquecido con OG estimula significativamente (45±8.2%, p<0.01) la proliferación celular a las 24 horas de incubación con osteoblastos. Las propiedades físicas y mecánicas del PMMA con OG incrementan sin alterar la sorción, solubilidad y porosidad. **Conclusión:** El OG solo o con PMMA tiene una biocompatibilidad aceptable, puede contribuir a la proliferación, regeneración celular y mejorar las propiedades físicas-mecánicas del PMMA.

Palabra Clave: *polimetil metacrilato; óxido de grafeno; materiales biocompatibles; propiedades mecánicas; bioensayo; proliferación celular.*

INTRODUCTION.

Graphene (G) is part of the group of carbon-based materials, which in turn is part of a family of various materials that have different manufacturing methods.[1] Graphene is a light and resistant material composed of two-dimensional lattices that are approximately less than 10 nanometers thick. These lattices are composed of carbon atoms united in a hexagonal honeycomb network.[2,3] Graphene oxide (GO) is the oxidized form of graphene, obtained from the oxidation of graphite.[4] There are different methods used in GO synthesis: mechanical exfoliation, which consists of breaking the interactions between each layer of graphene; chemical vapor deposition, a catalytic decomposition of hydrocarbons on the surface of a mechanical catalyst; and the Hummer method, which is a chemical technique where graphite is oxidized with concentrated sulfuric acid and other oxidants such as potassium permanganate ($KMnO_4$).[3,5]

Regarding the physical, chemical, and mechanical properties of GO, this is a rigid, almost transparent material that resists great forces and presents elasticity, thermal conductivity, impermeability, electrical conductivity, biocompatibility, and mechanical resistance.[3,5-7] Bases for denture must meet certain physical, mechanical, and chemical requirements. The physical requirements are:

1) to resemble the soft tissues of the oral cavity;

2) a high glass transition temperature (Tg) to avoid softening and deformation during use and cleaning;

3) good dimensional stability, so that the denture does not change its shape over time;

4) low solubility;

5) low water absorption;

6) low density, so that the prosthesis is light; and

7) high electrical conductivity, to maintain a healthy oral mucosa and to respond normally to hot or cold stimuli. The chemical requirements that denture base prostheses must meet are:

1) being chemically inert;

2) being insoluble in oral fluids; and

3) to not absorb water or saliva, as it can alter the mechanical properties of the material, as well as being unsanitary.[8]

In recent years, graphene oxide has had various applications in science and technology thanks to its physical and chemical properties. Its characteristics provide a whole world of applications for this material, such as the manufacture of touch screens for cell phones, the development of high-speed communications due to its excellent thermal and electrical conductivity, and its application in laser technology and materials processing due to its optical properties. However, for its biomedical application, it is necessary to determine the toxicity and physical properties that they can provide to biomaterials.[9]

Some of the applications of GO in biomedicine are its use in biosensors, devices for cellular imaging, and phototherapy for cancer treatment. In dentistry, GO is mainly known for its biocompatibility, and it is important to evaluate the capacities of its physical and chemical characteristics to ensure its safe and reliable use, since it has been shown that adding GO to biomaterials can potentially increase the properties of the material.[3,4,6] However, GO has not been fully characterized from the perspective of the biological impact of its applications in dentistry and *in vitro* with cultured oral mesenchymal cells.

The bioactive effect of graphene oxide has been evaluated on stem cells derived from human adipose tissue,[2] stem cells from the human periodontal ligament,[1] and stem cells from human dental pulp.[4] It was found that at a low concentration (<0.1mg/mL), GO did not show any cytotoxic effect, instead, it achieved cell proliferation and increased the expression

of several genes that are positively regulated with mineral-producing cells. On the other hand, the highest concentrations evidenced cytotoxicity. It is also reported that graphene and graphene oxide are cytotoxic at concentrations greater than 50 mg/mL.[6]

The aim of this study was to determine the cytotoxicity and effects on cell proliferation (1-30 days) of cultured human gingival fibroblasts (HGF), human dental pulp cells (HPC), and human osteoblasts (HBC); to determine the physical-mechanical-biological properties of poly (methylmethacrylate) (PMMA) enriched with graphene oxide through resistance and elastic modulus tests in flexion, sorption, solubility, porosity, and cytotoxicity tests in contact with HBC.

MATERIALS AND METHODS.

Characterization of graphite oxide (GO)

The powdered GO (Graphenemex, Mexico City, Mexico) was analyzed with scanning electron microscopy (SE Hitachi S5500, Tokyo, Japan). The sample was placed on carbon paper and analyzed at 200, 500, 1500 and 2500x, 20–25 kV acceleration, and at a pressure of 20–25 Pa in the vacuum chamber.

The size of the GO particle was determined from micrographs using ImageJ software (US National Institutes of Health, NIH, Bethesda, MD, USA).

Cell culture

The HGF, HPC and HBC cells were obtained from the cell bank of the Interdisciplinary Research Laboratory (IRL), Nanostructures and Biomaterials Area of the Escuela Nacional de Estudios Superiores, Unidad León, UNAM, Guanajuato, Mexico, with prior authorization from the research ethics committee (registration CE-16/004).

The cells were cultivated in modified essential medium (MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1,000 IU/mL of penicillin, 100mg/mL of Streptomycin (PenStrep, Sigma-Aldrich), and 1% L-glutamine (Glutamax, Life Technologies, Gibco, USA). The cells were incubated (37±1°C; relative humidity 95±1%; 5±1% CO_2) for 21 days (500±4 h) with changes of culture medium every third day. The cells were detached via treatment with 0.25% trypsin-EDTA (Sigma-Aldrich, USA).

Cytotoxicity and cell proliferation assay

Primary cultures of HBC, HPC and HGF were subcultured ($1x10^5$ cells/mL) for 48 hours in 96-well plates. The GO (Graphenemex, Mexico City, Mexico) was inoculated in the three types of cells at a dose of 0 to 0.5mg/mL and incubated for 1, 7, 14, 21 and 30 days at 37±1°C; relative humidity 95±1%; 5±1% CO_2. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) rapid colorimetric bioassay (0.2mg/mL) (Sigma-Aldrich, USA). The formazan crystals resulting after 7 hours of incubation were dissolved with DMSO (dimethylsulfoxide, Karal, León, Mexico).

Mitochondrial activity was determined with a microplate spectrophotometer (SkanGo, Fisher Scientific, Finland) at a 570 nm wavelength. Tests were carried out according to ISO-10993-5 Biological evaluation of medical devices —Part 5: Tests for *in vitro* cytotoxicity.

GO-enriched PMMA cytotoxicity

The primary culture HBCs were subcultured ($2x10^5$ cells/mL) in 24-well plates for 48±1 h in a humid atmosphere (37±1°C; relative humidity 95±1%; 5±1% CO_2) to achieve complete adhesion of the cells and adequate cell density. The GO-enriched acrylic samples were prepared with a 50±1x15±1x3.0±0.1mm dimension. They were stored in water at 37±1°C for 50±2 h to remove residual monomer. The samples were previously sterilized for 30±1 min in a UV sterilizer.

The cells were brought into direct contact with the acrylic samples and were incubated for 24±1h. Untreated culture wells were used as the control group. As mentioned above, the number of viable cells was determined by the MTT-rapid colorimetric bioassay.

Evaluation of the physical-mechanical properties of GO-enriched PMMA

Samples of thermosetting acrylic resin (thermo polymerizable OptyCril, NewStetic, Colombia) and acrylic resin with graphene oxide (PMMA-GO, Graphe Dental, Mexico) were prepared following the manufacturer's instructions (PMMA) to determine the mechanical (maximum stress or flexural strength, flexural elastic modulus) and physical (sorption, solubility, and porosity) properties according to ISO-20795-1: 2013 Dentistry —Base polymers— Part 1: Denture base polymers.

Flexural strength and elastic modulus

Ten 50±1x15±1x3.0±0.1mm samples were prepared and stored in water at 37±1°C for 50±2 h. After 50 hours, the sample was immediately placed on the bending support, which must be submerged in water at 37±1°C, according to ISO-20795-1:2013. There was a 5-minute wait before starting the test for the sample to reach the bath temperature.

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The samples were subjected to a three-point bending test, where they were placed on two supports separated by a distance (L). A force (F) was applied with a velocity of 1mm/min in the center of the sample using a Universal Mechanical Testing Machine (Instron 5567, Norwood, MA, USA) in order to deflect it. The samples were kept immersed in water at 37±1°C while applying the load (F in Newton) until fracture occurred. Flexural strength (R) values were obtained in MPa. The mathematical expression used considered a constant *p*-value of 32mm for L (distance between supports), width (b, in mm) and thickness (h, in mm):

$R = (3FL)/(2bh^2)$

For the calculation of elastic modulus, the following formula was used:

$E = (F'L^3)/(4bh^3d')$

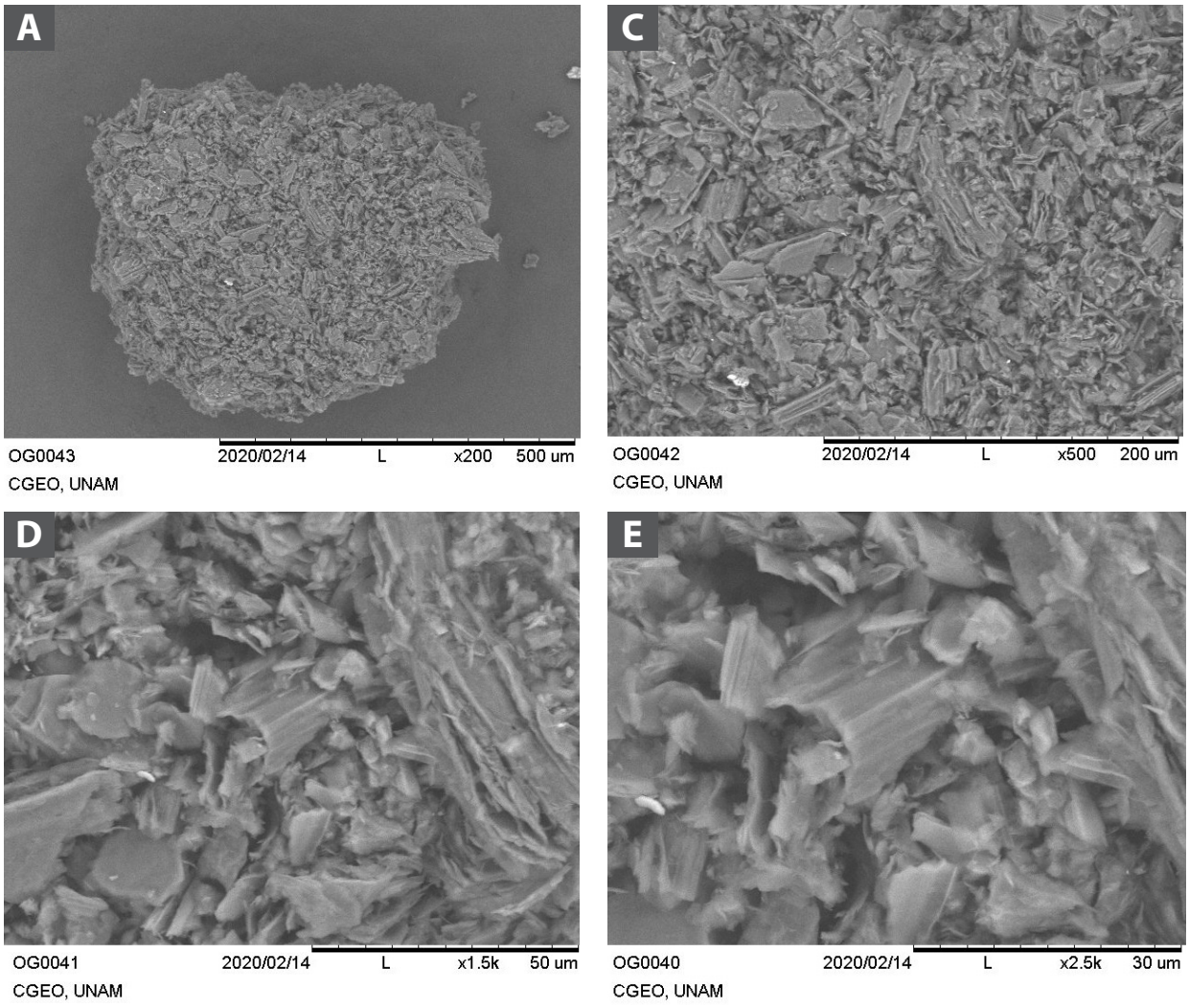
Where F' is the force in Newton at the yield point, with its corresponding deflection d', in mm.

Sorption and solubility

Following ISO-20795-1 guidelines, samples (n=10) of 50±1mm in diameter (D) and 0.50±0.10 mm in thickness (h) were prepared. The volume of each sample was calculated ($V=(3.1416D^2h/4)$. Samples were weighed on an analytical balance and then placed in a desiccator with freshly dried silica gel for 300±10 min at 130±5°C.

The desiccator was placed inside an oven at 37±1°C for 23±1 h. The samples were weighed again after this period had passed. This cycle was repeated until the difference in weight between two consecutive days was not greater than 0.0002g.

Figure 1. **Scanning electron micrographs of graphene oxide at different magnifications.**



A. 200x. **B.**500x. **C.**1500X. **D.**2,500x.
Samples were obtained at an acceleration of 20–25 kV with primary electrons. Particle size 66.67±64.76 µm and heterogeneous morphology.

This weight was reported as M_1. The samples were placed in water for 7 days (168±2h) at a temperature of 37°C±1°C. After that time, the samples were dried in open air and were then weighed. This weight was reported as M_2. The constant weight cycle was repeated until the difference of 0.0002g was achieved. This weight was reported as M_3.

Sorption was calculated as: $SOR = (M_2-M_3)/V$

Solubility was calculated as: $SOL = (M_1-M_3)/V$

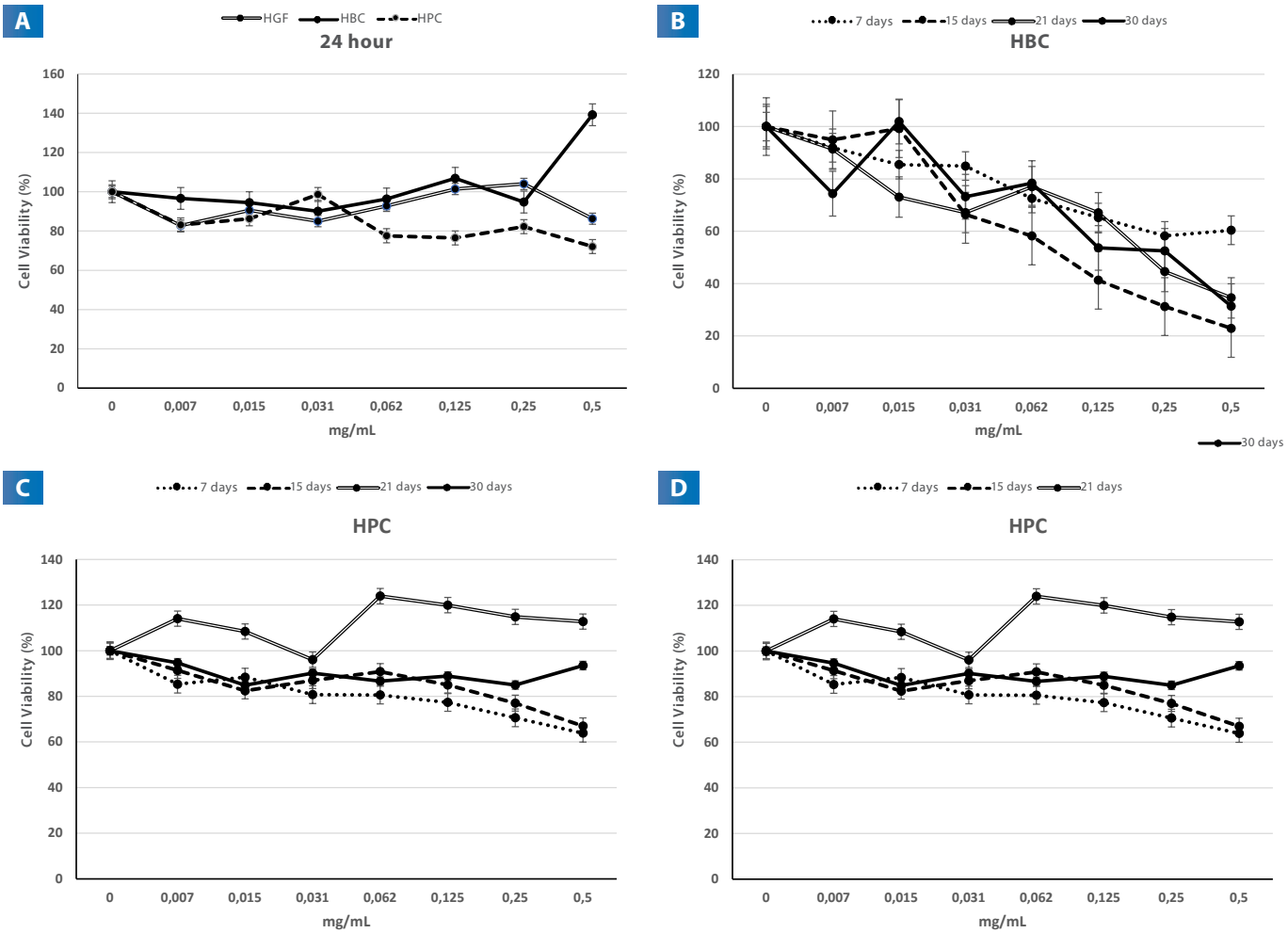
Porosity to the naked eye

The specimens used were the 50x15x3mm samples from the flex test before being stored in water. As ISO-20795-1: 2013 indicates, both the top and bottom 50x15mm sections of the sample surfaces were observed with the naked eye using natural sunlight.

Statistical analysis

The mean and standard deviation were calculated for the tests performed. The percentage of cell viability

Figure 2. Cytotoxicity of graphene oxide.



A. and its effects on cell proliferation of cultures of **B.** Human Osteoblasts (HBC). **C.**Human Gingival Fibroblasts (HGF) and **D.** Human Pulp Cells (HPC) at 24 hours, 7, 15, 21, and 30 days. Data were obtained in triplicate from three independent experiments (n=9).

Table 1. Cytotoxic effect of GO-enriched PMMA .

Group	Cell viability	Sample size and significance (t-Student)
Control	100.0±3.2	n=9
PMMA+OG	145±8.2%	n=9, $p<0.01$

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Table 2. Physical-mechanical properties of PMMA enriched with graphene oxide particles.

		Unit	PMMA	PMMA-GO
Physical tests	Pores	No.	0±0	0±0
	Sorption	g/mm^3	67±3	60±4
	Solubility	g/mm^3	0±0	0±0
Mechanical tests	Flexural strength	MPa	65±6	65±17
	Flexural modulus	GPa	2.79±0.51	2.66±0.32
	Flexural resilience	MJ/m^3	41±3	47±10
	Yield stress	MPa	37±9	40±20
	Yield strength	N	130±33	140±86
	Yield deflection	mm	1.11±0.30	1.17±0.52
	Energy to fracture	J	92	106

None of the physical and mechanical properties present statistically significant differences (p>0.5); n=10.

was determined from the cytotoxicity and proliferation curve. Shapiro-Wilks's normality tests and parametric tests (t-Student and Tukey post-hoc ANOVA) were used. The experiments were performed in triplicate to obtain reproducible data. Statistical significance was set with p-value <0.05 and a 95% reliability coefficient.

RESULTS.

Characterization of graphite oxide (GO)

Figure 1 shows the SEM micrographs of GO, where a heterogeneous morphology of particles with an average size of 66.67±64.76µm (range 15-200µm) can be observed.

Cytotoxicity and cell proliferation

Figure 2A shows the dose-response curve of GO in culture with HGF, HBC and HPC at 24 hours of incubation. According to the ISO-10993-5 specification (100-75% cell viability), it was deemed slightly cytotoxic, as follows: HPC<HGF<HBC, without reducing more than 75% of cell viability, which concludes that the compound is considered non-cytotoxic and even stimulates the proliferation of HBCs in a dose-dependent fashion.

Cell proliferation (Figure 2B, Figure 2C and Figure 2D) at 7, 15, 21, and 30 days shows a slight reduction in cell viability, with the sensitivity of cells being in the following order: HBC<HGF<HPC. This suggests that the compound is slightly cytotoxic.

Cytotoxicity of GO-enriched PMMA

Having GO-enriched PMMA in direct contact with HBC for 24 hours significantly increased cell viability by 45±8% (p<0.01) compared to the control group (Table 1).

The tested biomaterial shows adequate compatibility without altering cell viability, even increasing the number of human osteoblasts.

Physical-mechanical properties of GO-enriched PMMA

Table 2 shows the physical-mechanical properties of GO-enriched PMMA. The differences between both acrylic resins are significantly evident. The GO-enriched resin significantly decreased (p<0.05, t-Student) the elastic modulus, produced greater yield deflection, less aqueous sorption, equal number of pores and solubility, and greater resistance to bending. The samples showed a smooth appearance, without the presence of porosity and solubility.

DISCUSSION.

The evolution of biomaterials is due to the different variations that exist in terms of their chemical components and their origin, providing increasingly better properties that can be applied in various biomedical fields. However, one of the most important limitations for their use is the ability to be in contact with biological tissues without causing any type of damage. In the case of metal ions used in dentistry, it is important to determine the biocompatibility with the tissues of the oral cavity.

The studies on graphene and its derivatives have shown that they have significant biocompatibility, the-refore, they have become an excellent alternative in the scientific field for their application in different

areas, including dentistry. Graphene oxide is a relatively new material, and research on its applications is in its early stages.[10,11] Studies have shown that GO has zero or reduced cytotoxicity in some cell types such as human fibroblasts, mesenchymal stem cells, cells of the immune system, and human osteoblasts.[3] There are different factors that directly influence this effect, such as concentration, size, shape, and even the type of dispersant with which the GO particles are synthesized. Reports mention that functionally stable GO-based nanomaterials show less toxicity and modulate the differential behavior of these cells by increasing tissue adhesion, proliferation and regeneration.[12]

They have also been used as a guide in osteogenesis and adipogenesis.[9] Osteoblast adhesion is a requirement to initiate proliferation and for the synthesis of proteins (extracellular matrix proteins), morphogenetic factors, and osteoinductive molecules.[13]

After 48 hours of incubation, it was found that the osteoblasts that homogeneously covered the graphene oxide surface significantly increased proliferation. These results coincide with the proliferation obtained in our experiments at 30 days of incubation and with the significant increase in the proliferation of human osteoblasts in contact with GO-enriched PMMA at 24 h.

In addition to this, Olteanu *et al*.,[14] report that high concentrations (40 mg/mL) of GO reduce cell viability and alter the potential of certain cell organelles. In contrast, GO at low concentrations (4mg/mL) shows an adequate safety profile in human dental follicle stem cells.[13] In dentistry, graphene oxide has been used in biocomposites as a proposal for a new generation of composites due to its bactericidal effect against certain microorganisms. In tissue engineering, GO has been shown to have beneficial effects on osteogenesis and tissue regeneration.[11,13]

Denture bases must meet certain physical, mechanical, and chemical requirements. Regarding low water absorption,[9] in this study the solubility and sorption of PMMA with and without graphene oxide is zero. GO-enriched PMMA has less sorption than PMMA, which reduces both swelling and the possibility of microorganisms penetrating small cracks in the prosthesis surface, as well as the extraction of residual monomer.

Regarding mechanical properties, denture bases must have a high yield stress to ensure that the pressures generated when biting and chewing do not produce permanent deformations.

The GO-enriched PMMA registered 40 MPa, while the control group recorded 37 MPa, resulting in a 2.66 and 2.79 GPa elastic modulus, respectively. In turn, GO-enriched PMMA showed a yield deflection of 1.17mm, while the control group registered 1.11mm. Consequently, the resilience of GO-enriched PMMA is 47 MJ/m^3, while in the case of PMMA, it is 41 MJ/m^3. In other words, a low elastic modulus allows the prosthesis to be more flexible and withstand greater chewing forces. In the case of removable prostheses, if they were to fall and hit a hard surface there is a possibility of accidental damage or breakage, so these materials must have a high impact resistance.

A high resilience indicates that the impact force supported is greater and will allow a prosthesis made with GO-enriched PMMA to absorb more energy (106 J), which will significantly improve this property. Hence, for example, if the prosthesis were to fall to the ground when the patient is cleaning it, the probability of it breaking will be lower. Upper dentures invariably fracture mainly due to bending pressures or stresses, therefore, a key requirement in polymeric materials is high flexural strength.

Both materials, with and without graphene oxide, support 65 MPa. Additionally, denture fractures often occur due to fatigue mechanisms, in which the accumulation of small bending stresses after a period leads to the formation of small cracks that propagate, leading to fracture.[15]

Results of this study showed that GO-enriched PMMA meets the chemical requirements for denture base prostheses, as the samples have less water sorption than PMMA without GO.

GO-enriched PMMA porosity tests indicate that microbial adhesions are not feasible, reducing their probability of occurrence as there is no macroscopic evidence of pores that allow or stimulate microbial surface adhesion and proliferation. This behavior is in accordance with studies that conclude that, even without adding drugs or chemicals, GO-enriched PMMA exhibited better continuous anti-adhesive effects against microbial species for 28 days, compared to those exhibited by PMMA without graphene oxide by only increasing hydrophilicity.[16,17]

This suggests that GO-enriched PMMA can be used effectively in removable or temporary prostheses (resin for dental prostheses, orthodontic appliances, temporary

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resin) and biomaterials such as implants because they do not allow antimicrobial adhesion.

CONCLUSION.

Results from this study suggest that GO has good biocompatibility and it can contribute to cell proliferation and regeneration. Incorporation of GO into PMMA improved physical, mechanical, chemical, and biological properties in comparison to PMMA without GO.

These data suggest a potential use of GO for denture bases, contributing to their clinical and biological performance.

Conflict of interests: The authors declare no conflicts of interest.

Ethics approval: Approved by the research ethics committee of the Escuela Nacional de Estudios Superiores, Unidad León, Universidad Nacional Autónoma de México (UNAM), Guanajuato, Mexico (registration CE-16/004).

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Authors' contributions: García-Contreras: Bibliographic search; Physical and mechanical properties; Biological properties; Drafting the manuscript. Guzmán-Juárez: Bibliographic search; Sample preparation and characterization; Drafting the manuscript. López-Ramos: Bibliographic search; Sample preparation and characterization; Drafting the manuscript. Alvarez-Gayosso: Bibliographic search; Sample preparation and characterization; Physical and mechanical properties; Biological properties; Drafting the manuscript.

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