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Research Article

Analysis of *In-vivo* Cytotoxicity and Irritability of an Epoxy Nanocomposite

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ABSTRACT

Objectives: To assess a high-performance epoxy nanocomposite through the analysis of *in vivo* accumulated irritability on the oral mucosa, and cell viability through *in vitro* cytotoxicity/biological reactivity in mouse cells.

Materials and Methods: Nanocomposite samples were manufactured in epoxy resin Araldite GY 260 using the hardener Aradur HY 951, a diluent, and carbon nanotubes previously dosed as reinforcement. The samples were then used in the tests.

Results: Cell viability test showed cellular growth above 70%, and an irritability index of 0.00, which is considered ideal for materials used with humans.

Conclusion: The sample was considered non-irritating to the oral mucosa and non-cytotoxic, rendering its use viable in dental prostheses.

Keywords: Carbon nanotubes; Prostheses and implants; Composites; Nanotechnology; Epoxy; Polymers; CAD-CAM

Abbreviations: DM: Diabetes Mellitus; HTN: Hypertension: COPD: Chronic Obstructive Pulmonary Disease

INTRODUCTION

In dentistry, techniques and materials are continuously evolving. Epoxy resins are easy-to-process polymers, with good chemical and thermal resistance and high adhesion. Carbon fibers [1] and carbon nanotubes [2,3] have been added to epoxy resins aiming to improve their physical and mechanic properties.

Carbon nanotubes have been regarded as essential materials in nanomedicine with great potential as drug carriers, transfection agents, and scaffolds in tissue engineering [4]. However, their application is limited due to their toxicity relative to surface area and reactivity [5]. Besides, high concentrations of nanoparticles can generate reactive oxygen species within the cells, which can lead to DNA mutation. On the other hand, they showed no signs of toxicity against human epithelial cells *in vitro* [6].

In this sense, some authors claim that synthetic carbon nanostructures need to be treated to increase biocompatibility and generate cell responses. Hence, a bioactive carbon with natural functionalization is needed in order to trigger cell adhesion with future cell proliferation without the need for additional chemical or biological treatments [7]. This results in less toxicity in cell cultures, thus increasing its potential for biological applications [6].

Therefore, it is key to assess a high-performance epoxy nanocomposite through the analysis of *in vivo* accumulated oral mucous irritability and cell viability through in vitro cytotoxicity/biological reactivity tests in mouse cells.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the Dentistry Research Center São Leopoldo Mandic (2016/0691).

The samples were supplied by the Department of Physics of the Federal University of Minas Gerais (UFMG), holder of a patent (PI# ND040) for an innovative method that allows the scale production of carbon nanotubes (CNT) by arc discharge [4]. They also supplied the high-performance nanocomposites used in this study that are based on preliminary studies and protected by a deposited patent (BR 1020140161813).

The epoxy resin chosen to manufacture the nanocomposite in this study was Araldite GY 260 with the hardener Aradur HY 951, both manufactured by Huntsman (Huntsman Brasil Química Ltda, São Paulo, SP, Brazil). 99.5% pure acetone (Acetone PA, PM 58,08,

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batch: 88236, Labsynth Produtos para Laboratório Ltda, Diadema, São Paulo, Brazil) was used as diluent.

CNT used as reinforcement phase were dosed (Mettler Toledo, mod. AT 201, d=0,01 mg, Switzerland) to the desired composite dispersion concentration. They were then stored in glass containers along with alumina spheres of 2 mm in diameter and ethyl alcohol (MW 46.07, Batch: 15499/08. Cromaline Química Fina Ltda, Diadema, São Paulo, Brazil); the ensemble was submitted to spin stirring at 25 rpm overnight (U.S. Stoneware, East Palestine, OH 44413). Then, CNT were filtered to separate the alumina phase and stored in a 70°C oven (Bluem-Ashewille NC-USA) to remove the ethyl alcohol.

After drying, the CNT was stored in metal containers and treated with liquid nitrogen for 7 minutes applied to the surface. Acetone at 10% m/m (relative to epoxy resin mass) was added to the container and the ensemble was submitted to sonication for 10 minutes (Fisher Scientific, mod. FS 220, 500 W, and 20 kHz). The epoxy resin was then poured into the CNT/acetone solution and submitted to an ultrasound bath for 3 hours.

The solvent is removed from the CNT/epoxy/acetone mixture by heating it to 50°C for 5 hours followed by exposure to exhaustion for 5 hours and a new ultrasound bath for 1 hour before adding the hardeners in a 5:1 ratio (epoxy:hardener, m/m) that was manually homogenized for 1 minute. The mixture was poured into the molds (13 mm in diameter for 4 mm in thickness) and cured in exhaustion for 24 hours at room temperature.

The epoxy nanocomposite samples were cut into 5 gram pieces; the pieces were washed in running water, dried, packed, and autoclaved to be used in the tests described in the following.

In vivo accumulated oral mucous irritability test (CTFA, 2007)

In vivo accumulated oral mucous irritability test was conducted by Medlab DIAGNOSTIC PRODUCTS LTDA, conducted according to reference CTFA (Cosmetic, Toiletry, and Fragrance Association)-Technical Guidelines-CTFA Safety Evaluation Guidelines, 2007. With the aim of assessing the irritant potential of the substance in direct contact with the oral mucous (Figure 1).

Characterization: Species: *Rattus norvegicus* (rats); lineage: Wistar; sex: females; 10 healthy individuals aging 1.5 months; origin: Anilab, Paulínia-SP.

The inclusion criteria were healthy and free from infection individuals. The exclusion criteria were individuals with no health issues, as per previous blood tests.

Figure 1: Oral mucous irritability test (Source: Own authorship).

The animals were kept according to the regulations and laws in effect, ensuring their integrity and welfare.

Sample preparation: the sample was extracted at 121°C for 1 hour in a Shaker incubator at 100 rpm in the ratio of 0.2 g of the sample to 1.0 mL of 0.9% sodium chloride solution. The extract was used up to 24 hours after preparation. The resulting liquid (extract) was homogeneous and colorless.

The individuals were randomly selected and assigned to one of two groups: test and control (n=5). The oral mucous was examined and only individuals with no signs of lesions were used in the test. Bodyweight was taken and registered at the beginning and end of the test.

The sample extract was applied topically with the use of a cotton swab that was kept in direct contact with the mucosa for 30 seconds. The procedure was repeated 5 days a week for 28 days. A 0.9% sodium chloride solution was applied in the control group following the same procedure described for the sample.

The oral mucous was assessed for local effects (edema and erythema) 24 hours, 48 hours, and 72 hours after the last application.

Analysis of *in vitro* cytotoxicity/biological reactivity (ISO 10993-5: 2009)

The test was conducted at the Laboratório Biosintesis® P and D do Brasil-Laboratory for Research, Development and Innovation in Biocompatibility and *in vitro* Biological Studies.

This test aimed to assess the test substance named 668X-epoxy nanocomposite using a cytotoxicity test.

The test substance, weighing 4.83 g, was exposed to UV light for 15 minutes on each side for sterilization. The sample was then used in the preparation of an extract with a concentration of 0.2 g/mL by adding 24.15 mL of DMEM without dilution.

Preparation of reference substances

Negative control (PEAD): The reference substance was used to prepare an extract in the concentration of 0.2 g/mL, which was used with no dilutions.

Positive control-Natural Latex (NL): The reference substance was to prepare an extract in the concentration of 0.2 g/mL, which was used with no dilutions.

For all substances, the incubation time and the extract preparation was followed by the recommendations on the document POP_TEC_002-PSE: preparation of substances for studies. The study was conducted on cells derived from the ovary of Chinese hamsters CHO-KI (ATCC CCL-61) because they are a permanent cell line, commercially available, well-established, and easy to cultivate. The cells were kept in culture according to the procedure described in POP_TEC_003-MCC: Cell culture maintenance, until use. The cells were exposed to the extract or test substance solution and/or reference substance for approximately 24 hours.

At the end of the test, the plates were read at the wavelength of 490 nm according to POP_TEC_001-ECPC: Cytotoxicity and Cell Proliferation Tests (Figures 2-4) to calculate the percent cell viability (CV %).

RESULTS

Accumulated oral mucous irritability test results (CTFA, 2007) (Tables 1 and 2)

For categorical analysis, p value was calculated using both the

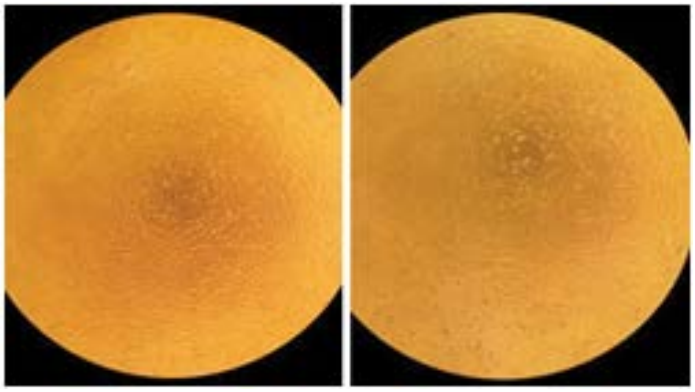

Figure 2: Before and after PEAD (Source: Own authorship).

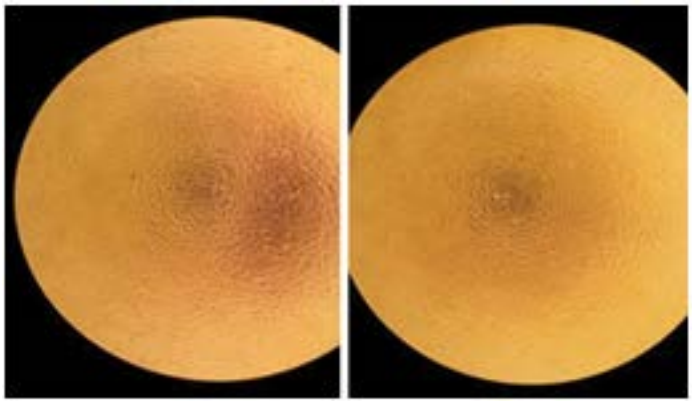

Figure 3: Before and after TL (Source: Own authorship).

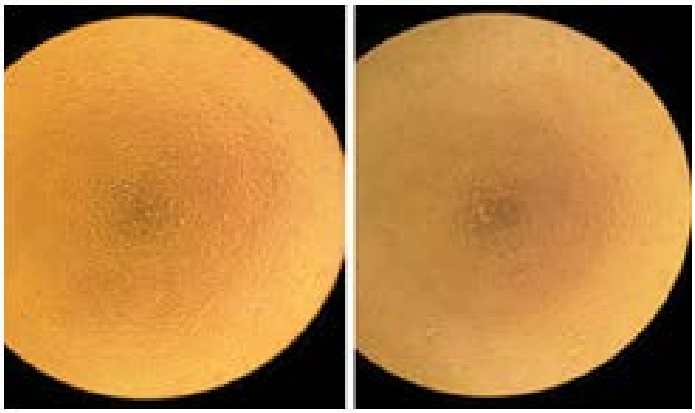

Figure 4: Before and after-test substance (Source: Own authorship).

Pearson's Chi Square Test and the Fisher's Exact Test. p value <0.005 was considered significant (Table 1).

Caption: Edema and Erythema: 0-None; 1-minor (slightly distinct); 2-Well-defined erythema/Mild edema (well-defined edges); 3-Moderate erythema/Moderate edema (very distinct, approx 1 mm of swelling); 4-Severe erythema (dark red) to the occurrence of lesions/Severe edema (more than 1 mm of swelling, extending beyond the exposed area).

The categories of irritability (index value/classification) were distributed as: 0-0.49 non-irritating, 0.50-1.00 mildly irritating; 1.01-2.00 moderately irritating; and >2.00 severely irritating (Medlab, 2018).

In vitro cytotoxicity/biological reactivity results (ISO 10993-5: 2009) (Table 3)

The cells exposed to the test substance showed cell viability above 70%.

DISCUSSION

With the advent of nanotechnology, among the various types of nanomaterials, Carbon Nanotubes (CNT) may be the most representative [8]. Therefore, assessing their cytotoxicity and the potential damage of their use to human beings and the environment is as important as understanding their physical and chemical properties [9]. In this sense, nanotoxicology plays a key role in analyzing the consequences of the effects of nanodevices and nanostructures on biological systems.

Studies report that further studies on the cytotoxicity and irritability of nanocomposites are still needed [10]. Some point out that primitive nanotube, with physical similarities to asbestos-a highly toxic substance when inhaled-can also cause inflammation in humans [5]. Corroborating this suspicion, a study analyzed the pulmonary pathology profile of mice repeatedly exposed to carbon nanotubes or crocidolite and showed that both substances led to granulomatous inflammation and an increase in interstitial collagen [11].

Other studies focus on the physical characteristics of CNT, such as dimensions and surface properties, and how it relates to its toxicity. These studies showed that the myeloperoxidase enzyme, which is found in human neutrophils, can degrade single-walled carbon nanotubes, thus preventing inflammation in mice lungs [12].

On this line of investigation, improved bio-composites were administered intraperitoneally to enhance any potentially harmful

Table 1: Test group (Source: Medlab, 2018).

Test system			1	2	3	4	5	
Weight (g)		Initial	146.65	145.21	134.88	131.14	137.67	Averages
		Final	213.52	234.31	206.25	194.12	205.76	
Periods	24 h	Edema	0	0	0	0	0	0.0
		Erythema	0	0	0	0	0	0.0
	48 h	Edema	0	0	0	0	0	0.0
		Erythema	0	0	0	0	0	0.0
	72 h	Edema	0	0	0	0	0	0.0
		Erythema	0	0	0	0	0	0.0
Total								0.0
Mucous Irritation Index (Total /3)								0.00

Table 2: Control group (Source: Medlab, 2018).

.5			1	2	3	4	5	Averages
Weight (g)		Initial	150.48	132.07	140.36	133.01	155.73	
		Final	220.88	201.17	227.24	209.86	229.97	
Periods	24 h	Edema	0	0	0	0	0	0.0
		Erythema	0	0	0	0	0	0.0
	48 h	Edema	0	0	0	0	0	0.0
		Erythema	0	0	0	0	0	0.0
	72 h	Edema	0	0	0	0	0	0.0
		Erythema	0	0	0	0	0	0.0
Total								0.0
Mucous Irritation Index (Total /3)								0.00

Table 3: Average cell viability and standard deviation for test and reference substances [Source: Biosintesis® (2018)].

Substance	Cell viability (%)	Cell viability (%)
PD01 (PEAD)	108.3	2.9
LT02 (Natural Latex)	20.1	2.1
668X (Test substance)	107	3.8

effects; neither cardiovascular effects were found nor adverse effects on metabolic activity due to the possible release of toxic components [13]. Furthermore, the combination of multiple-walled CNT with other chemical components offers new opportunities for the development of polymer nanocomposites with biosimilar properties. Another important evidence of the lack of toxicity of these materials is the growth and proliferation of osteoblasts [14].

CNT used in the present study are produced by the Department of Physics of the Federal University of Minas Gerais (UFMG), holder of a patent (PI#ND040) for an innovative method that allows scale production of carbon nanotubes via arc discharge [4]. The high-performance nanocomposite samples used in this study, based on preliminary studies and protected by a deposited patent (BR 1020140161813) have been produced in the same institution.

A series of systematic studies on the cytotoxicity of colligated and functionalized nanomaterials showed that multilayered carboxylated carbon nanotubes above the concentration of 50 µg/mL alter cell viability. However, subsequent analysis showed that carbon nanotubes coupled with plasmids (DNA-MWCNTs) in concentrations between 5 µg/mL and 20 µg/mL lacked cytotoxicity *in vitro* and that the biocompatibility of nanocomposites and the efficiency of carbon nanotubes purification showed no toxicity or negative effects against Vero cells; also with biocompatibility, the growth of the chondrocytes was stimulated [15].

A cytocompatibility study showed proliferation of osteoblasts and favorable cell adhesion with excellent biocompatibility and cytocompatibility when MWCNTs are incorporated into bioactive nanocomposites of carbon nanotubes and Polyether Ether Ketone (PEEK) [15,16]. The present study also showed favorable laboratory results to the epoxy nanocomposite evaluated since it showed no local edema or erythema in vivo, and was deemed non-cytotoxic in the *in vitro* test since cell viability remained above 70% upon exposure to the extract (ISO 10993-5:2009).

Considering the scenario described and that epoxy nanocomposite is a new and promising material, further toxicology, resistance, and behavioral tests are needed to shed light on the material's potential.

CONCLUSION

According to the criteria of classification of the method, the accumulated oral mucous irritability test sample was considered non-irritant.

According to the result presented by the laboratory Biosintesis®, the test substance 668X-epoxy nanocomposite was classified as non-cytotoxic.

It is possible to conclude that the epoxy nanocomposite analyzed here can be used in dental prostheses.

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