Cytotoxicity Evaluation of a Hydroxiapatite-reinforced Polymeric Resin using Human Peripheral Blood Mononuclear Cells (PBMCs)

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ABSTRACT

The use of hydroxyapatite-reinforced composite materials in the medical field has considerably increased in recent years because of the high biocompatibility and bioactive properties of this material, especially when it is used in bone replacement applications. It is imperative that new materials are throughly analyzed to ensure biofunctionality. This study describes the response of human peripheral blood mononuclear cells (PBMCs), separated from leukocyte packages obtained from healthy donors, in contact with a 90-10 weigth % composite manufactured from an isophthalic polyester resin and reinforced with HAp and calcium triphosphate particles. The viability of PBCMs in the presence of this novel composite was evaluated every 24 hours for 5 days using a commercial luminescent cell viability assay (Premix WST-1 Cell Proliferation Assay), positive and negative controls were used. PBMCs are part of the immune system and are usually stimulated by antigens and mitogens. Although the viability assessment demonstrated that HAp-reinforced resin does not have a cytotoxic effect on the PBMCs, the high cell proliferation values obtained via ELISA could be due to a mitogenic or antigenic effect of the composite. Because of the elevated cell proliferation, it was needed to confirm that the material was not acting as a mitogen. To do this, yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay with the cell line 231 (breast cancer cells) was performed. The viability of this 231-cell line was not affected by the presence of the composite. Based on the results, a pro-inflammatory mediator production assessment should be performed.

EXPERIMENTAL PROCEDURE

Before any new materials are incorporated into artificial implants, it is essential to ensure their biocompatibility and, when possible, their bioactivity. Therefore, new materials must undergo rigorous testing before they can be used. This study focused on the cytotoxic analysis of an isophthalic resin reinforced with hydroxyapatite and calcium triphosphate particles. The effect of the on human peripheral blood mononuclear cells (PBMCs) was evaluated for five days. PBMCs were isolated from leukocyte packages obtained from healthy donors and donated by the blood bank from the Hospital Militar de Especialidades para la Mujer y Neonatología, Mexico City, Mexico. The cells were isolated by density gradient centrifugation with Lymphoprep™, washed three times with RPMI 1640 and placed 5ml of X-VIVO™ culture medium. A suspension of 1 x 10^6 cells/ml was prepared for the viability assay and cells were cultured at a concentration of $5x10^4$ cells per well in a 96-well plate. The cells were incubated in the presence of the composite in two forms; one set was exposed to 5mm-circular plates from 1 to 5 days, while the other set was exposed to different doses of the composite in particle-form for 24h and 48h. For the dose response, PBMCs were exposed to different concentrations of the composite ranging from 10 µg to 100 µg as micro and nanoparticles. The cells were incubated in the presence of the material at 37 ° C in a humidified atmosphere maintained at 5% CO_2. Cell viability was assessed by ELISA with WST-1 titer every 24 hours. Briefly, the tetrazolium salts dissociate by cellular enzymes into formazan; an expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This increase in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates with the number of metabolically active cells in culture.

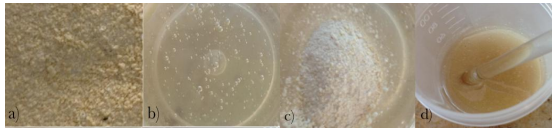


Fig. 1 Material Preparation: a) Hydroxyapatite and calcium triphosphate particles, b) Isophthalic resin, c) Mixture and d) Addition of curing agent

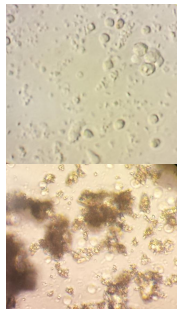
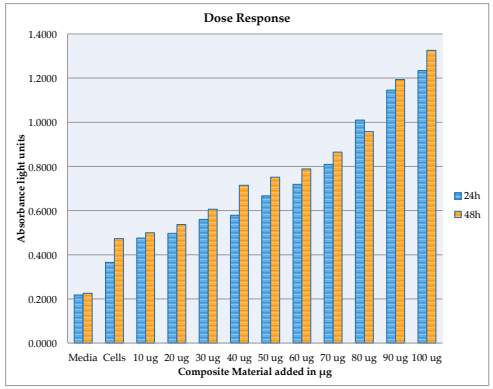
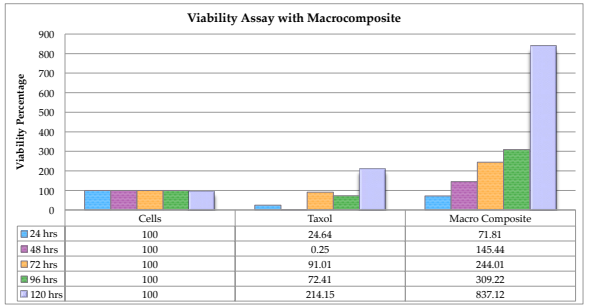
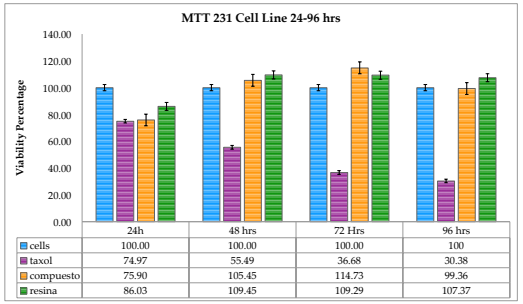


Fig. 2. Top. PBMCs at 48h. Bottom. PBMC at 48hrs with a dose concentration of 100µg of composite





	Cells	Taxol	Macro Composite
24 hrs	100	24.64	71.81
48 hrs	100	0.25	145.44
72 hrs	100	91.01	244.01
96 hrs	100	72.41	309.22
120 hrs	100	214.15	837.12



	24h	48 hrs	72 Hrs	96 hrs
cells	100.00	100.00	100.00	100
taxol	74.97	55.49	36.68	30.38
compuesto	75.90	105.45	114.73	99.36
resina	86.03	109.45	109.29	107.37

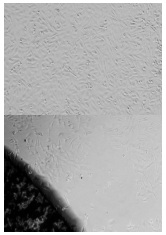


Fig. 3. Top. 231 cell line at 72 h. Bottom. 231 cell line at 72hrs in presence of composite

RESULTS

As shown by the graphs, PBMCs displayed higher viability than the negative control; the higher the treatment concentration, the higher the proliferation. There was no increase in the viability in concentrations of 90 µg and 100 µg. Proliferation was more significant between 96 and 120 hours of exposure. PBMCs exposed to this material exhibited a 8-fold viability value compared to negative controls. It was concluded that the composite material is not cytotoxic. Mononuclear cells are part of the immune system and a high cell proliferation may be due to the presence of a mitogen or an antigen. The results of this study showed that these cells are over stimulated by the presence of the composite. Therefore, it was necessary to confirm that the material was not mitogenic. An additional MTT assay with cell line 231 was performed. Cancer cells were exposed to the isophthalic resin and the composite for 96 hours. MTT analysis was performed every 24 hours to assess the cellular response. Results from MTT assay are evidence of possible antigenic response in PBMCs since the 231-cell line did not proliferate excessively and cell viability was not affected.

CONCLUSIONS

There is an extremely high and abnormal proliferation of the PBMCs exposed to the studied material. Exposure of the 231-cell line to the material demonstrated no mitogenic activity from the material. Consequently, the composite might act as an antigen and, therefore, when implanted in a biological system frustrated phagocytosis or chronic inflammation in tissues adjacent to the implant could occur.

REFERENCES

1. Krecher, C.R. et al. (2203) CD4+ and CD8+ Cells in Cryopreserved Human PBMC Maintain Full Functionality in Cytokine ELIPSPOT Assays. Journal of Immunological Methods. 278(1-2):79-93
2. Miro-Mur, F. et al. (2008) Medical-Grade Silicone Induces Release of Proinflammatory Cytokines in Peripheral Blood Mononuclear Cells Without Activating T Cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. DOI: 10.1002/jbm.b.31312
3. Martinesi, M. (2006) Biocompatibility Evaluation of Surface-treated AISI316L Austenitic Stainless Steel in Human Cell Cultures. *Journal of Biomedical Materials Research Part A*. DOI 10.1002/jbm.a
4. Boyum, A. (1968) Separation of leucocytes from blood and bone marrow Scand. J. Clin. Lab. Invest., 21, suppl.97
5. ATTC. Primary Peripheral Blood Mononuclear Cells (PBMC), Normal, Human (ATCC® PCS-800-011™) http://www.atcc.org/products/all/PCS-800-011.aspx.
6. Coligan, John E. (1999) *Current Protocols in Immunology*. John Wiley & Sons, Inc.
7. Lee, Jae-in et al. A Photoelastic Stress Analysis of Screw-and Cement-Retained Implant Prostheses with Marginal Gaps. *Clinical Implant Dentistry and Related Research*. (2013) **15** (5); 735-749
8. Introduction to Stress Analysis by the PhotoStress® Method. Tech Note TN-702-2, Document Number: 11212. Vishay Precision Group, 2011, (www.micro-measurements.com)
9. Ausiello, C.M. et Al. (1986) Proliferation of Human Peripheral Blood Mononuclear Cells Induced By Candida Albican and its Cells Wall Fractions. *Journal of Medical Microbiology*. 22(3):195-202

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