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Cytotoxic and genotoxic effects of the exposure of human lymphocytes to a calcium phosphate cement *in vitro*

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ABSTRACT: *The cytotoxic and genotoxic effects of a phosphate bone cement on human peripheral lymphocyte cultures exposed for 48 hr were determined. The mitotic index and the integrity of the DNA molecule were used as biocompatibility markers. It was found that the cement was not cytotoxic, but genotoxic at concentrations of ≥100 µg/ml. The cement was also characterized using scanning electron microscopy (SEM) and X-ray diffraction (XRD). (Journal of Applied Biomaterials & Biomechanics 2005; 3: 29-34)*

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INTRODUCTION

Ceramics based on calcium phosphate compounds have been widely used in clinical practice, especially in orthopedics and odontology. This is due to their high biocompatibility, their easy manipulation and adaptation (1-5). Dos Santos et al (6) underlined the importance of *in vitro* biocompatibility testing, as these assays can provide fast results concerning the interactions of materials in the biological media. They support that *in vitro* experiments should precede the *in vivo* assays without forgetting that the first may not represent the real situation of an implant. Nevertheless, there have been comparative studies in which different calcium phosphates degrade in the same way, both *in vitro* and *in vivo* (1).
Most biocompatibility assays are performed following the American Standard for Testing Materials (ASTM) recommendations, which include the use of monolayer cell cultures as experimental systems. Human peripheral blood lymphocytes have been widely used as an experimental model because of their availability, they are easy to culture, results can be obtained within short periods (3 days) and several end-points can be registered. Their natural physiological conditions are preserved *in vitro* since whole blood is placed in the culture flask (7). Lymphocytes circulate in the blood, recognize antigens, produce antibodies and are primarily responsible for cell-mediated immunity. Therefore, peripheral lymphocytes can be found everywhere within the organism, and they accumulate together with molecules to eliminate any antigenic agent from the body. This characteristic makes them very useful as testing systems for possible biomaterials, since they will also be subject to the possible toxic effects of the material.
Cell deformation and flask detachment are generally used as damage markers in monolayer cultures. Cells within a population can differ in susceptibility and it is possible that some of them will survive the exposure to a given material, unless it is extremely toxic or lethal. Complementary endpoints should then be incorporated to determine whether these

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surviving cells bear some non-lethal modification, which could alter the stability of the biological system (i.e. altered cell cycle kinetics, chromosomal alterations, DNA damage, etc).

One parameter that provides information about the degree to which a given material is toxic to living cells (cytotoxicity) is the mitotic index. It represents the proliferation fraction in a cell population (7, 8) and is determined by the proportion of cells that are in mitosis. At the molecular level, damage to the DNA molecule can also be detected. The single cell gel electrophoresis, or comet assay, is a very sensitive method to test genotoxicity. When one of the two DNA strands is broken at one or more points along its length, the genetic material shows a different electrophoretic migration pattern. Damaged nuclear DNA is visualized as a comet with its head and tail. The length of the tail is a direct function of the induced damage (9-13).

A calcium phosphate cement was produced to be used as bone filler material, to provide anchorage or fixation to one device in the living organism for as long as required. This cement can be prepared in the form of a paste that can be set, which can be easily introduced into the defective bone using a syringe. Its components react to produce hydroxyapatite (HA) as the product of the cement formation reaction in the presence of water. However, it has been observed that the paste disintegrates partially or completely or exhibits a non-homogeneous behavior as soon as it is exposed to body fluids or other aqueous solutions (14).

There are no reports concerning the toxicity of this phosphate cement in the literature. Therefore, it is very important to determine its biocompatibility. In this study, we demonstrate the cytotoxic and genotoxic effects of this material on human peripheral lymphocyte cultures exposed for 48 hr. In addition, we characterized this cement using scanning electron microscopy (SEM) and X-ray diffraction (XRD).

MATERIALS AND METHODS

Calcium phosphate bone cement preparation

The cement samples were composed of HA (HA = $Ca_{10}(PO_4)_6(OH)_2$) at 5wt%, calcium carbonate ($CaCO_3$) at 5wt% and α-tricalcium phosphate (α-TCP = a-$Ca_3(PO_4)_2$) at 90wt% (15).

Calcium phosphate bone cement characterization

Scanning electron microscopy. The microstructure of the calcium phosphate bone cement was studied using SEM. The sample was covered with a gold layer and examined under a Leica Cambridge Steroscan 440 microscope. This was carried out to give complementary information regarding the morphology of the powder.

X-ray diffraction. The cement was analyzed by XRD using a D5000 X-ray-diffractometer (Siemens) with Cu Kα radiation 35 KV and 30 mA. The angular interval 2θ analyzed was from 2° to 70° with 2°/min for the qualitative analysis. JCPD files were used to identify the different calcium phosphate compounds of the cement.

Biocompatibility tests

Peripheral human blood lymphocyte cultures served as the experimental model to determine if the cement was either cytotoxic or genotoxic or both. The experimental protocol followed was based on that described by Preston et al (16).

Bone cement suspensions. Stock aqueous suspensions of the phosphate cement were prepared at concentrations of 100, 1000 and 2000 µg/ml. They were sterilized by autoclave.

Cell cultures. Peripheral blood samples were obtained from six healthy, adult donors (three females and three males). Cultures were prepared according to Arakaki and Sparkes (17), placing 0.3 ml of whole blood in plastic tubes (NUNC) containing 2.25 ml of McCoy 5a culture medium (GIBCO BRL) added to phytohemaglutinin (GIBCO BRL) and a commercial antibiotic solution, antibac (Microlab). The cultures were incubated at 37 °C.

Treatment. After the first 24 hr of incubation at 37 °C, 0.25 ml aliquots from the stock cement suspensions were added to each culture tube to obtain the final concentrations of 10, 100 and 200 µg/ml of culture medium. The same volume of sterilized distilled water was added to the control cultures. Cultures were then re-incubated at 37 °C for a further 48 hr. The three experimental groups and the controls were prepared in triplicate (i.e. three tubes for each condition). One hour before cell harvesting, colcemid (Microlab) was added to the cultures to arrest cells in metaphase. Cell harvesting consisted of a hypotonic treatment with KCl 0.057 M for 20 min followed by fixation with Carnoy's fixative. Slides were stained with 10% Giemsa (Merck) (18).

Cytotoxicity test. The proportion of cells in metaphase was determined from a total number of 6,000 cells per treatment per donor.

Genotoxicity test. Before adding colcemid, an aliquot of 150 µl was taken from each culture flask and washed twice with PBS. These samples were used to perform the comet assay following the

Singh et al procedure (9). Briefly, it consisted of preparing agarose gels containing the cells on fully frosted microscope slides (Fisher). The slides were immersed in a lysing solution for at least 1 hr at 4 °C. They were then transferred to a horizontal electrophoresis box, left in the freshly prepared alkaline buffer for 20 min and exposed to an electric current of 25 V, 300 mA, for 20 min. After careful washing with neutralization buffer, the slides were stained with ethidium bromide and kept in the dark overnight at 4 °C. Three hundred nuclei per set for each donor were analyzed for DNA migration and measured using a fluorescent microscope (Olympus).

RESULTS AND DISCUSSION

Scanning electron microscopy. The initial microstructure of the material showed a homogeneous powder with particles between 1-10 µm (Fig. 1). The majority of particles had a rounded shape and others were polyhedrical, although this shape gradually changed in aqueous solution. The rounded HA particles have proved to be the less harmful to human monocytes *in vitro* (5).

X-ray diffraction. Figure 2 shows the X-ray spectrum of the cement powder. HA, whitlockite and α-tricalcium phosphate XRD lines were observed with a quantitative composition of 9, 15.3 and 75.7%, respectively. Similar phosphate cements to the one described here have been developed by other research groups (19-21).

Cytotoxicity. Table I shows the mitotic index values standardized through the root square method (22) for a better comparison. Variations in the response to the conditions of culturing was observed among donors, a phenomenon that has also been reported by other authors and interpreted as natural individual variability (8, 23-25). From the six donors, only those control cultures from donor 5 showed significant differences from its respective experimental ones (Tukey, $p<0.05$). This behavior accounts for the different sensitivity among individuals in a population. In spite of the observed variability, the average mitotic indexes registered in the experimental cultures did not differ from the controls (multiway factorial analysis of variance, blocked and repeated measures experimental designs, $p>0.05$ (22) performed with the statistical STATA 2000 software) (26). Based on these results, it was evident that the bone phosphate cement was not cytotoxic since the cultures exposed to it grew satisfactorily (i.e. cells did not die) and cell proliferation was unaltered, it was not suppressed or restrained nor incremented.

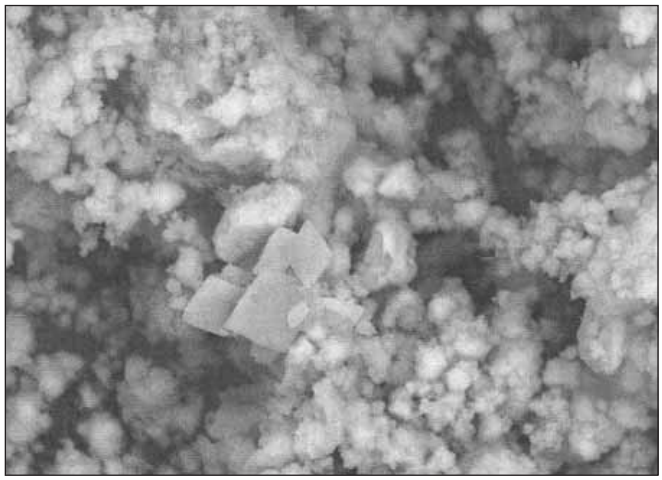

Fig. 1 - *Microstructure of the bone cement.*

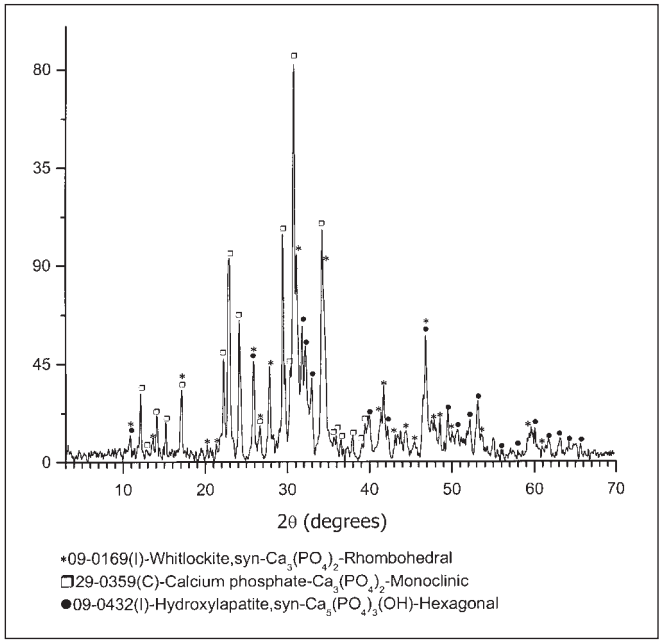

Fig. 2 - *Diffraction spectrum of the bone cement.*

Genotoxicity. Table II presents the results obtained from the comet assay. It was observed that the percentage of damaged cells tended to increase as a function of the ceramic concentration. The two higher concentrations of bone cement significantly increased this cellular fraction. The average DNA migration registered in each culture set demonstrated the same behavior: DNA migration at bone cement concentrations of 100 and 200 µg/ml was longer (Tab. III) and the differences between them were significant (Tukey, $p<0.05$). These results indi-

TABLE I - MEAN MITOTIC INDEX ± STANDARD ERROR (%) IN LYMPHOCYTE CULTURES EXPOSED TO SUSPENSIONS OF A CERAMIC MATERIAL (STANDARDIZED DATA USED)

Donor	**Set**			
	Control	**10 µg/ml**	**100 µg/ml**	**200 µg/ml**
1	0.41 ± 0.09	0.43 ± 0.10	0.65 ± 0.10	0.51 ± 0.08
2	1.07 ± 0.08	0.78 ± 0.04	0.79 ± 0.04	0.77 ± 0.12
3	1.28 ± 0.06	1.32 ± 0.02	1.18 ± 0.06	1.21 ± 0.08
4	0.94 ± 0.11	1.28 ± 0.06	1.09 ± 0.05	1.03 ± 0.07
5	1.96 ± 0.16	1.44 ± 0.04	1.42 ± 0.13	1.28 ± 0.07
6	1.22 ± 0.10	0.91 ± 0.09	1.19 ± 0.04	1.19 ± 0.05
Average	**1.15**	**1.03**	**1.05**	**1.00**
SE	**0.21**	**0.16**	**0.12**	**0.12**

TABLE II - PROPORTION OF CELLS WITH DNA DAMAGE DETERMINED BY THE COMET ASSAY

	Set			
	Control	**10 µg/ml**	**100 µg/ml**	**200 µg/ml**
Average (%)	38	35	43*	48*
SE	7.22	14.50	17.63	19.75

*Significant difference with regard to the control group (Tukey; $p<0.05$)

TABLE III - AVERAGE DNA MIGRATION IN LYMPHOCYTES CULTURES EXPOSED TO THE CERAMIC SUSPENSION FOR 48 hr

	Set			
	Control	**10 µg/ml**	**100 µg/ml**	**200 µg/ml**
Average (%)	55.02	55.31	65.56*	74.96*
SE	1.12	1.25	1.37	1.25

*Significant difference with regard to the control group (Tukey; $p<0.05$)

TABLE IV - PROPORTION OF CELLS DISTRIBUTED IN INTERVALS OF DNA MIGRATION LENGTH AFTER EXPOSITION TO THE PHOSPHATE CEMENT

DNA migration length (µm)	**Frequency (%)**			
	Control	**10 µg/ml**	**100 µg/ml**	**200 µg/ml**
1-20	3	0	10	2
21-40	35	25	37	24
41-60	39	44	38	37
61-80	18	22	12	14
81-100	2	9	4	19
>101	2	0	0	4

cated a genotoxic effect of the material at those concentrations. DNA damage can be induced by direct interaction or indirectly. This last mechanism seems to be the most probable for the bone phosphate cement studied, since any cell with cement particulates inside was observed under the microscope as occurred with other materials (for example, zeolite; Aguilar et al, unpublished data). There-

fore, one of several molecules derived from the cement-physiological environment interactions could be the toxic agent. The distribution of DNA migration length ranged from 1 to >100 µm (Tab. IV). Statistical analysis of this distribution was performed using the Kruskal-Wallis test, which confirmed that the only concentration of bone cement that was not genotoxic was 10 µg/ml ($k=158.56$; $p>0.05$).

Considering that in clinical practice the cement would be used to restore damaged bone structures, it seems difficult to imagine that the amount of fine particles that could be released in body fluids as residual debris could reach concentrations as high as those tested in this study. Nevertheless, the possibility of small circulating particles and their toxic potential should not be discarded.

Further bioassays should be performed to accurately determine the genotoxic potential of this new calcium phosphate cement *in vitro* as well as *in vivo*.

CONCLUSIONS

Phosphate bone cement is not cytotoxic, but at concentrations of ≥100 µg/ml, it induces DNA damage. Based on our observations, it is suggested that one or several compounds formed during the interaction of the cement with the physiological medium could be the genotoxic agent. The human lymphocyte culture was demonstrated to be a suitable experimental system to test biocompatibility in the less sensitive cellular fraction.

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