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Cytotoxicity Evaluation and Crystallochemical Analysis of a Novel and Commercially Available Bone Substitute Material

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Alloplastic biomaterials are an alternative for autologous transplants and xenografts in oral surgery and dental implantology. These non-immunogenic and resorbable materials are becoming the basis for complete and predictable guided bone regeneration in many cases. The chemical composition of a great majority of them is based on calcium phosphate salts. *In vivo* performance is often variable.
Objectives. The objective was to evaluate the biological and chemical properties of an experimental bone substitute material.
Material and Methods. The present research focuses on the cytotoxicity comparison and physiochemical characterization of two biomaterials: a novel chitosan/tricalcium phosphate/alginate composite (CH/TCP/Ag) and a commercially available synthetic bone graft made of HA (60%) and βTCP (40%) (HA/TCP). The materials were evaluated according to PN-EN ISO 10993 Biological evaluation of medical devices i.e. cytotoxicity on mouse fibroblasts (L929) and, in addition, tests on human osteoblasts (hFOB1.19) and human osteosarcoma (MG-63) were conducted. The crystallochemical analysis was performed using the X-ray powder diffraction method. The Bruker-AXS D8 Advance diffractometer (Karlsruhe, Germany) was used to collect diffractograms.
Results. The tested materials showed a close resemblance in chemical composition and a considerable differentiation in cytotoxic response.
Conclusions. The novel composite demonstrated a high degree of cytocompatibility, which is promising in future clinical trials (**Adv Clin Exp Med 2015, 24, 3, 511–516**).

Key words: bone substitute material, chitosan, guided bone regeneration, X-ray powder diffraction, cytotoxicity.

Guided bone regeneration (GBR) has significantly changed implant dentistry in the past 20 years. The lack of bone in the alveolar ridge is a great challenge to the success of the rehabilitation of the stomatognathic system. Hard tissue augmentation is becoming more common as is usage of bone substitute materials in oral surgery. Biomaterials for stimulating osseous regeneration should combine osteogenic, osteoconductive and osteoinductive properties. They should also resorb and be replaced by newly formed bone [1–3]. As many studies have demonstrated, although autografts are the most suitable bone augmentation materials, their limited availability in the oral tissues and their high rate of post surgical morbidity has motivated investigators to utilize other biomaterials in bone regeneration [2–7].

Alloplastic bone substitute materials have attracted more attention in this field over the last few years as an alternative for autologic transplants

and xenogenic materials. These non-immunogenic and resorbable biomaterials are the basis for complete and predictable guided bone regeneration.

The chemical composition of the great majority of them is based on calcium phosphate salts. The biocompatibility and osteoconduction properties of e.g. hydroxyapatite (HA) are well documented. The *in vivo* performance of particular brands of alloplastic grafts is often variable [3, 8].

Those with a low Ca/P ratio resorb more rapidly, which may cause unpredictable biodegradation profiles and decrease of mechanical strength. On the contrary, those with greater Ca/P ratios degrade gradually or not at all and may give rise to remnants that can induce inflammation [2, 8]. In addition, lower degradation rates usually result in less new bone formation around the scaffold. The chemical composition alone is by no means the only factor in determining the nature and extent of scaffold degradation. Besides the physiological conditions, characteristics such as crystallinity, particle size, porosity, surface roughness, cytocompatibility and level of impurities have been reported to influence the biological performance [2, 3, 8].

Therefore, the research for model alloplastic bone grafts is in progress. It focuses e.g. on biodegradable polymer-based materials with the ability to do controlled delivery of drugs, materials which can be combined with growth factors, injectable, sculptable, fast-setting biomaterials.

Our project is in line with the recent trends in designing such an osteoconductive, "user-friendly" bone graft which is perfectly tolerated by human tissues for guided bone regeneration. The evaluation of the cytotoxicity in comparison to commercially available synthetic bone graft is argued herein. It is undoubtedly a critical step in the further development of synthetic biomaterial for tissue engineering applications. Additionally, a crystallochemical analysis of both materials using the X-ray powder diffraction method complements our comparison.

Material and Methods

The evaluated biomaterial was designed as a diphase system, where the solid phase consisted of chitosan (CH)/β-tri-calcium phosphate (TCP) particles and the liquid one of a 2% solution of alginate salt [4, 9]. The injectable system formation relied on calcium ions being released from chitosan/TCP particles into the liquid alginic phase, which tends to form a gel in the presence of calcium ions. Our material was based on chitosan, whose use has been widely accepted in bone tissue engineering [6, 9, 10]. The second component was β-TCP, in contrast to HA, a fully resorbing, synthetic grafting material [2].

Chitosan (~95% degree of deacetylation) [9, 11] was purchased from Medical Heppe GmbH, β-tricalcium phosphate from Sigma Aldrich and alginic acid sodium salt from brown algae was purchased from Fluka. The SEM images of the particles are shown in Fig. 1. The detailed synthesis of the composite was described in previous publications [7].

The control material was a commercially available synthetic bone graft made of HA (60%) and βTCP (40%), 4Bone Synthetic Bone Graft (Biomatlante, France). The granule size of the chosen samples was 1–2 mm, which was comparable to the CH/TCP/Ag.

Our material was evaluated according to the European/Polish Standard PN-EN ISO 10993 Biological evaluation of medical devices.

The first step of the toxicity evaluation was a mutagenicity analysis. The evaluation of the mutagenic action of an extract was carried out on the basis of the reference Ames test according to PN-EN ISO 10993-3:2009 Biological evaluation of medical devices. The results of the genotoxicity, carcinogenicity and reproductive toxicity tests were published in 2012 [7].

Tests for *in vitro* cytotoxicity of both biomaterials were performed according to the European/ /Polish Standard PN-EN ISO 10993-5 tests for *in vitro* cytotoxicity. All methods we used were accepted by the PCA (Polish Center for Accreditation – certificate No. AB 774) and EDQM (European Directorate for the Quality of Medicines – attestation No. MJA 032). We used the mouse fibroblast L929 cell line recommended by PN-EN ISO 10993-5 guidelines for this study.

Cytotoxicity tests were also performed with hFOB1.19 (human osteoblasts) and MG-63 (human osteosarcoma). The cell lines were purchased from the American Type Culture Collection. It is worth mentioning that human osteoblasts are not very often chosen for the bone grafting materials evaluation. They are rather difficult to isolate, culture and maintain. Until recently, the ability to study this model was limited.

The L929 and MG-63 cells were cultured in T-25 culture flasks using Minimum Essential Medium Eagle (EMEM, Lonza**,** USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotics containing penicillin (100 UI/mL), streptomycin (100 μg/mL), amphotericin (250 ng/ /mL), 2 mM glutamine (Lonza, USA), and non-essential amino acids. The cells were incubated at 37°C, 95% relative humidity and 5% CO_2.

The hFOB1.19 cells were cultured as above but in Dulbecco's Modified Eagle Medium, i.e. Nutrient Mixture F-12 (DMEM/F-12) (Gibco,

Invitrogen, USA) supplemented with 10% (v/v) FBS at 34°C, 95% relative humidity and 5% CO_2.

A monolayer of the cells tested was routinely obtained within 3–5 days of incubation until the cells reached 80–90% confluency. Confluent cells were detached from the culture flask with 0.25% (w/v) trypsin EDTA 200 mg/L (Lonza, USA). After counting with a cell coulter (Coulter Z2, Beckman Coulter, USA), singularized cells were resuspended in culture media at a density of 1×10^5 cells/mL and then seeded into 96-well tissue culture plates at a concentration of 1×10^4 cells/well (100 µL). The cells were incubated for 24 h before cytotoxicity testing.

The tests were performed on extracts of leachable products from the tested materials. The extraction conditions were set according to PN-EN ISO 10993-5:2009 guidelines, i.e. each of the tested materials was incubated in culture media with serum at 37°C for 24 h (0.1 g/mL or 0.2 g/mL depending on the form and type of the material). After that time, the culture media from 96-well plates were aspirated and the material extracts and the extracts of the negative and positive controls were added to the cultured cells in the plates (100 µL/well) for 24; 48 or 72 h. The cell cultures treated only with the media were used as a control. The culture media alone were used as blanks. The cytotoxicity of the leachable products was determined with the EZ4U cell proliferation and toxicity assay (Biomedica GmbH, Austria). The EZ4U assay is based on water soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide), which is converted into water soluble colored salt by succinate dehydrogenase, acting only in the mitochondria of living cells.

The cells were incubated with media alone or with extracts for 24, 48 or 72 h. Then, 20 µL of EZ4U was directly added to each well after preparation of that reagent according to the manufacturer's protocol. The plates were further incubated for about 2 h at 37°C (L929 and MG63) or at 34°C (hOFB1.19).

The number of viable cells positively correlated to the color intensity of the formed salt was determined by spectrophotometric measurements at 560 nm and 650 nm.

The cell viability was reported as % of the control cultures. To diminish the background interference, the final absorbance was calculated by the subtraction: $A_{560nm} - A_{650nm}$ for each well. A_{blank} was regarded in all absorbance measures.

Experimental data is expressed as mean ± standard deviation (SD) from at least 6 experiments. The data was analyzed using SigmaPlot v. 11 software. Statistical analysis was performed using the one-way ANOVA with a subsequent Tukey *post-hoc* test. Values of $p < 0.05$ were considered to be statistically significant.

The crystallochemical analysis of both materials was performed using the X-ray powder diffraction method. XRPD is a useful tool to identify phase and composition features and evaluate the crystallinity of the materials. Moreover, it provides information concerning the chemical composition and the major functional groups. The powder samples after grinding in an agate mortar were placed in sample holders and then on an X-ray diffractometer. A Bruker-AXS D8 Advance powder diffractometer (Karlsruhe, Germany) was used to collect diffractograms. The parallel monochromatic CuKα X-ray beam coming from a Göbel

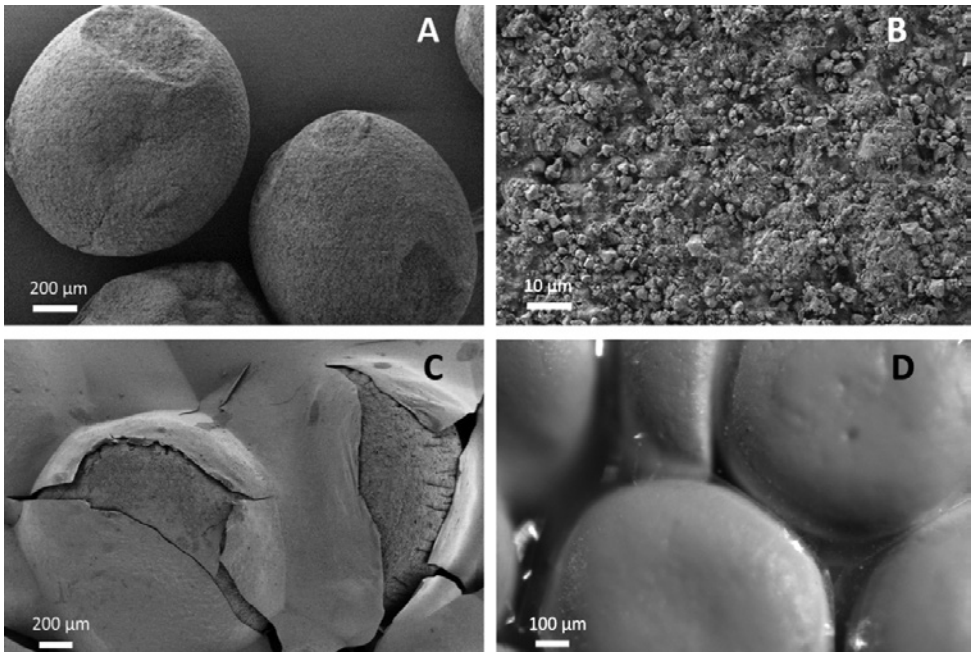

Fig 1. Morphology and topography of CH/TCP particles (A, B) and CH/TCP/ /Ag composite (C, D) obtained by SEM analysis [7]

mirror and the θ-θ scan mode were applied. The data was collected in 2θ range from 3 to 80° with a 0.02°/s scan rate. The collected diffractograms were then treated with standard smoothing and background subtracting procedures.

Results

L929 Viability

The cytotoxic effects of the leachable products from the tested materials are shown in Fig. 2 and 3. Both tests fulfilled the criteria of negative (NC) and positive control (PC) response according to the guidelines. Viability of cells under the negative control was above 90% in every stage of exposition time and the under positive control from 65 to 8%. Viability of cells after 24, 48 and 72 h of exposition in the presence of the extracts of the commercial material (HA/TCP) amounted to a range between 114 and 96% and in the presence of the experimental material (CH/TCP/Ag) to a range between 108 and 106%. According to PN-EN ISO 10993-5:2009, both biomaterials were not cytotoxic. However, CH/TCP/Ag seemed to stimulate proliferation of L929 cells slightly after 24 h (Fig. 2).

MG-63 Viability

This test also fulfilled the criteria for negative and positive control responses. Viability of cells in negative control maintained about 100% for each time point and decreased from 80 to 31% between 24 and 72 h in the positive one. However, there were significant differences between both tested materials. The viability of cells after exposure to CH/TCP/Ag at all tested time points varied from 109 to 94% and for HA/TCP from 89 to 55%. According to the PN-EN ISO 10993-5:2009, the latter one can be reported as cytotoxic and CH/TCP/Ag as non-cytotoxic material for MG-63 cells. The results are shown in Fig. 3.

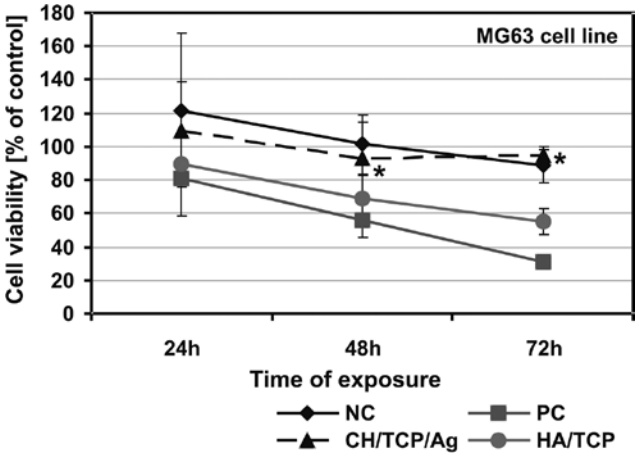

Fig. 3. Survival of human osteosarcoma cells. Each point represents the mean ± SD. (n ≥ 8), asterisks indicate significance at $p < 0.05$, for comparison with HA/TCP

hFOB1.19 Viability

This test also fulfilled the criteria for negative and positive control response, i.e.: above 80% of viable cells for each time point in negative (NC) and from 52 to 22% of viable cells between 24 and 72 h in positive control (PC) were observed. The viability of hFOB1.19 after 24, 48 and 72 h of exposure to CH/TCP/Ag ranged from 66 to 100% and for HA/TCP from 78 to 31%. Moreover, the CH/TCP/Ag seemed to stimulate proliferation of the cells by ca. 30% between 48 and 72 h of exposure, as was shown in Fig. 4.

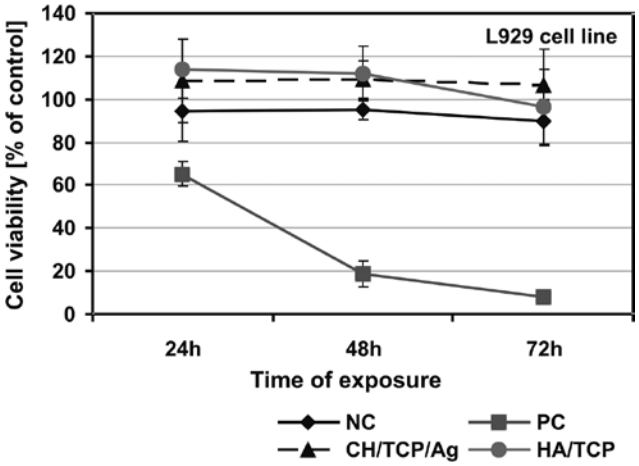

Fig. 2. Survival of mouse fibroblast cells. Each point represents the mean ± SD. (n ≥ 6), asterisks indicate significance at $p < 0.05$, for comparison with HA/TCP

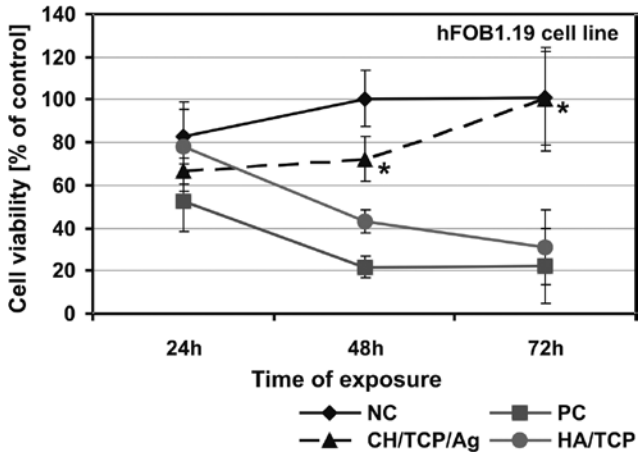

Fig. 4. Survival of human osteoblast cells. Each point represents the mean ± SD. (n ≥ 8), asterisks indicate significance at $p < 0.05$, for comparison with HA/TCP

According to PN-EN ISO 10993-5:2009, HA/TCP can be described as cytotoxic and CH/TCP/Ag as non-cytotoxic material for hFOB1.19 cells.

Crystallochemical Analysis

The comparison of the diffraction patterns for the experimental (depicted in black) and commercial (depicted in red) materials is shown in Fig. 6. Partial qualitative interpretation of the results was performed using the pdf-2 data base (International Centre for Diffraction Data, 2005). All diffraction picks for the experimental material correspond to the calcium phosphate structure (Ca_3 $(PO)_4$ – Whitlockite, syn, shown as the dark-blue vertical lines). The analysis of the diffraction pattern of the 4bone (HA/TCP) material led to the conclusion that an additional phase, apart from calcium phosphate, was present. This additional phase can be interpreted as one of the hydroxyapatites (the schematic diffraction pattern is shown in the form of green vertical lines). The results are consistent with the manufacturer's declaration.

Discussion

Cell cultures and bacterial strains provide a convenient, controllable and repetitive instrument for a preliminary evaluation of the biological response. Cytotoxicity and genotoxicity are important factors affecting the systemic compatibility of an implantable material. In general, cytotoxicity *in vitro* is a simulation of the biological response to the material through the exposure of the cell cultures to the extracts or direct contact to the material. Due to serious and life-threatening consequences, these tests are gaining increasing public interest [12].

The results obtained in the cytotoxicity test with L929 were favorable, according to expectations and the clinical application. If we had finished the investigation at this point, we could confirm the legitimacy of the cytocompatibility of both materials. They were non-cytotoxic according to the appropriate EN ISO norm.

Nevertheless we wanted to widen our experiment, testing more cell lines which could be more sensitive or susceptible to the extracts of bone grafts.

The results of the tests performed on the MG-63 and hFOB1.19 cell lines were somewhat different as regarded both materials. Both cell lines were not negatively affected by the extracts of CH/TCP/Ag. On the contrary, the response of both cell lines unquestionably proved the cytotoxicity of HA/TCP. In addition, CH/TCP/Ag showed stimulative properties for the proliferation of osteoblasts. Although hFOB1.19 and MG63 are cell lines not often used

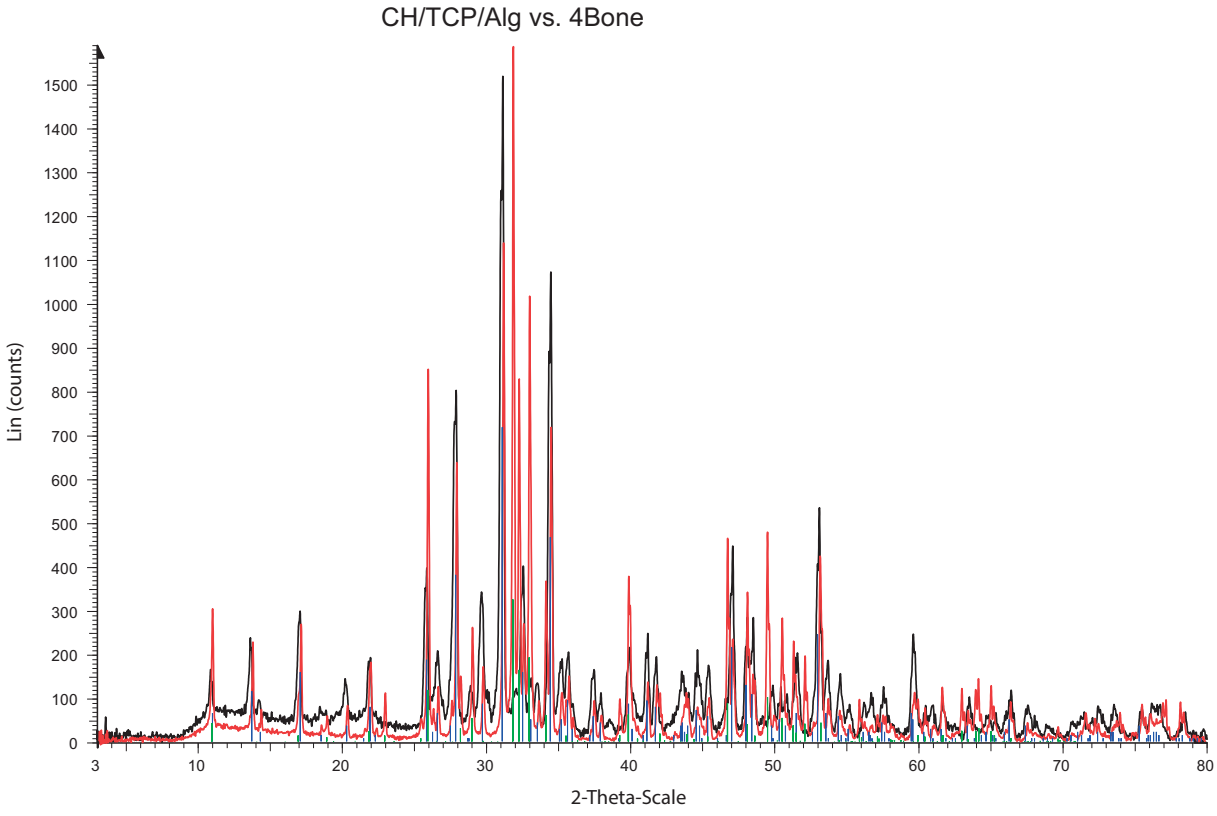

Fig. 5. CH/Tcp/Ag, HA/TCP (4Bone). $Ca_3(PO)_4$ – Whitlockite, syn, calcium sodium dysprosium phosphate hydroxide

for evaluating biomaterials, our results seem to be valuable and worth noting.

Searching for answers to the diversification of cytotoxicity tests, we performed the instrumental crystallochemical analysis. The results of the XRPD analysis were rather predictable and consistent with the manufacturer's statement. Sintered calcium phosphate ceramics, particularly TCP and HA, are inorganic graft materials often used in oral surgery. The rationale for their use is found in their biochemical and structural similarity to the mineral phase of bone. Both materials are available as solid or porous block, chips, or granules. Each has markedly different physical and degradative properties, strongly dependent on the structure (solid or porous), microstructure and the presence of impurities.

In spite of the acknowledged excellent biocompatibility of calcium phosphate ceramics-based materials [1–3, 5], the present *in vitro* study demonstrated some differentiation between chemically similar biomaterials. This may be the reason of the variable clinical performance, but we are not in command of *in vivo* comparison at the moment.

The results of the cytocompatibility tests of our new, alloplastic, injectable bone substitute were encouraging, according to expectations and the biological properties of the basic materials. Less positive results of the commercially available bone graft confirm the need for an evaluation of the medical devices authorized for marketing by independent laboratories.

The previous trials confirmed our ability to manufacture a bone grafting material with precisely definable physical and chemical properties and consistent batch quality. The preclinical evaluation we performed on the new, alloplastic, injectable bone substitute opens possibilities to patent pending and advanced *in vivo* testing.

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