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Original paper

Formation and preclinical evaluation of a new alloplastic injectable bone substitute material

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Alloplastic bone substitute materials are raising some more interest as an alternative for autologic transplants and xenogenic materials especially in oral surgery over the last few years. These non-immunogenic and completely resorbable biomaterials are the basis for complete and predictable guided bone regeneration. In the majority of cases, such a material is chosen because of its convenient application by surgeons.

The main objective of our project was to design and fabricate an osteoconductive, injectable and readily tolerable by human tissues biomaterial for guided bone regeneration. For this purpose, a self-setting composite consisting of chitosan/tricalcium phosphate microparticles and sodium alginate was made. The material obtained was characterized by microsphere and agglomerate morphology and microstructure. Its features relating to setting time and mechanical properties were precisely investigated. Our material was also evaluated according to PN-EN ISO 10993 *Biological evaluation of medical devices*, i.e., the in vitro tests for genotoxicity and cytotoxicity were conduced. Then, the following examinations were performed: subchronic systemic toxicity, skin sensitization, irritation and delayed-type hypersensitivity and local effects after implantation.

The material tested showed a high degree of cytocompatibility, fulfilled the requirements of International Standards and seemed to be a "user friendly" material for oral surgeons.

Key words: *alginate, bone substitute material, chitosan, guided bone regeneration, β-tricalcium phosphate*

1. Introduction

Modern dentistry largely depends on current technologies, and dental implantology is the medical field with great demands for bone substitute materials. Although autogenous bone has always been the golden standard, due to being the most biologically viable material, its harvesting is a real challenge. For some time now, a large number of osteoconductive xenogenic and synthetic substitutes have been available.

However, the use of the term "bone substitute material" is highly questionable in the case of hydroxyapatite materials. The materials made of bovine or equine bones, processed or partially synthetic, do not resorb totally. They can act as volumetric fillers and scaffolds

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but are not replaced by newly formed bone. Another problem arising from the use of the materials of biological origin is the need to inform patients of residual immunological and infection risks [1].

It seems most likely that for these reasons alloplastic bone substitute materials attract more attention as an alternative for autologic transplants and xenogenic materials in oral surgery over the last few years. These non-immunogenic and entirely resorbable biomaterials are the basis for complete and predictable guided bone regeneration. In the majority of cases, the method of choice for such a material is its convenient application for surgeons.

The main objective of our project was to design and fabricate an osteoconductive, injectable and well tolerated by human tissues biomaterial for guided bone regeneration. The evaluation of the physical and biological properties is discussed herein.

2. Materials and methods

The biomaterial tested was designed as a biphasic 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 [2], [3]. The injectable system was developed as follows: calcium ions were released from chitosan/TCP particles into the liquid alginic phase where they induced gel formation. Our material was based on chitosan, whose use has been widely accepted in bone tissue engineering [4]–[6]. The second component was β-TCP, in contrast to CH, fully resorbed, synthetic grafting material [1].

Chitosan (~95% degree of deacetylation) [4], [7] was purchased from Medical Heppe GmbH, β-tri-calcium phosphate from Sigma Aldrich and alginic acid sodium salt from brown algae was purchased from Fluka.

Chitosan/β-tri-calcium phosphate beads were prepared by hydrodynamic forming CH/TCP slurry at the rate of drop formation and collected in a precipitation bath consisting of a NaOH solution. Chitosan/β-TCP solution was prepared by suspending TCP powder in a 2% acetic acid solution containing 2% of chitosan. The homogeneous suspension was placed in 50 ml syringe (BD Perfusion) and pressed out through the plastic nozzle using an infusion pump (AP22, Ascor) into a continuously stirred (rpm = 800) 2% NaOH bath. Afterwards, the formed beads were rinsed with distilled water and dehydrated with the use of 96% ethanol. Then the granules were dried at a room temperature and immersed in a $CaCl_2$ solution in order to enrich the CH/TCP beads with Ca^{2+} ions. In this way, an inorganic phase could become a carrier and a source of the cross-linking agent released into alginate gel-like structure. The injectable system was obtained by mixing CH/TCP beads with the liquid alginate in 1:2 mass ratio. Calcium chloride concentration and immersion time were considered and studied in the hereby presented studies.

The morphological examining the particles of CH/TCP composite and the formed injectable system was performed under optical microscope (Nikon Eclipse 80i) and by SEM analysis (Zeiss Supra). The gelation time of the injectable system was evaluated with the use of a double plunger syringe. In order to determine the setting time, the liquid alginic phase was forced out by the composite granule phase which was previously placed in the syringe. The system was assumed to be gelled when resistance of the liquid phase flow increased preventing alginate transport by the beads and was measured as the setting time with a timer. To conduct the investigation 1.00 g of composite granules was sucked into a syringe and 2 ml of alginic acid sodium salt was added. The influence of the calcium chloride concentration (1, 2, 10%) and the time of soaking the CH/TCP phase in the solution of calcium chloride on the morphology of composite particles was evaluated during the study.

The mechanical properties concerning the compression tests were studied on the samples cylindrical in shape. Two types of specimens were tested: those previously dried at room temperature for 24 h (n = 6) and the samples tested in their hydrated form, immediately after material formation (n = 6). The universal electromechanical testing set-up, MTS Q/test 10 with work parameters: 10 kN load and a constant speed of crosshead displacement equal to 0.1 mm/s was used. Young modulus values were calculated from the slope of the linear part of the stress–strain curves, and the compressive strength was assessed from the first maximum of stress visible on the curves.

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

In the first step of the toxicity evaluation, the material mutagenicity and cytotoxicity were analzyed. The mutagenicity of the extract was evaluation on the basis of the reference Ames test according to PN-EN ISO 10993-3:2009 *Biological evaluation of medical devices*. *Tests for genotoxicity, carcinogenicity and reproductive toxicity*. The test consists in evaluating the ability of the compound studied to induce reverse mutation in cells of auxotrophic *Salmonella typhimurium* strains (TA97, TA98, TA100, and TA102). The

bacterial cells, in which reverse mutation occurred, showed the ability to reproduce and form colonies on the minimal medium lacking growth factor (histidine).

Tests for in vitro cytotoxicity of the material were performed according to the European/Polish Standard PN-EN ISO 10993-5. *Tests for in vitro cytotoxicity*. All the methods used have been accepted by PCA (Polskie Centrum Akredytacji – certificate No. AB 774) and EDQM (European Directorate for the Quality of Medicines – attestation No. MJA 032).

Mouse fibroblasts NCTC clone 929 in the logarithmic phase of growth were trypsinized, and resuspended in MEM supplemented with 10% FBS (foetal bovine serum), 1% antibiotics (streptomycin, penicillin and amphothericin) and glutamine. Then the 96- or 6-well plates were inoculated with the cells at the proper density, depending on the kind of the test used, and cultivated at 37 °C in 5% CO_2 atmosphere. After 24 h, the cells were exposed for 48 hr to the extract of the material prepared in MEM with serum for 24 hr or to the direct contact with the material for 24 hr. To confirm the results obtained with direct contact and to eliminate the effect of possible mechanical damage to the cells which could occur in that kind of test the cytotoxicity was performed also by agar diffusion. The results obtained were interpreted on the basis of the PN-EN ISO 10993-5:2009 and the internal procedure accepted by PCA.

Thereafter, the following in vivo examinations according to EMEA and EN ISO 10993 Part 10 and Part 11 were carried out:

- subchronic systemic toxicity (test was performed on mice),
- skin irritation and intracutaneous reactivity (on rabbits),
- irritation and delayed-type hypersensitivity (on guinea pigs).

The test protocols were approved by the Local Ethical Commission for Animal Experimentation.

The last step of the biological evaluation was the test for local effects after implantation. It was performed according to EN ISO 10993-6:2007, to assess the biological response of bone tissue to implanted material. The selected specie was the Wistar rat. 45 mature male rodents, weighing 300–400 g, were used for the study. 8.5-mm diameter bone defects were prepared in calvaria bone under i.v. general anaesthesia and filled with the tested biomaterial, commercially available β-TCP/PLA bone substitute, or left unfilled. Histological analysis was performed 1 month and 3 months after implantation. The calvaria was removed and immediately fixed in 10% buffered formalin. After decalcification (with Decalicifier II – Surgipath), the specimens underwent routine histological processing using paraffin-embedding method. The sections (4–5 μm thick) were stained with hematoxilin and eosin and examined under light microscope (Nikon Eclipse 50i microscope).

3. Results

The particles obtained by the drop forming method are shown in figure 1A, B, C. Due to the incorporated inorganic calcium phosphate phase they possess a rough surface with well-developed microstructure, which can be found by considering SEM microphotographs presented in figure 1C. Formed into cylindrical shape, previously dried, CH/TCP/Alg biomaterial was also analyzed by SEM and the pictures obtained are presented in figure 1. When compared with CH/TCP beads, the alginic layer seems to possess a smooth surface that tends to be fractured when the material completely dries. Figure 1A, C, D presents the photographs obtained under optical microscope; from the left hand side, respectively, the morphology of CH/TCP microspheres (A) and the formed injectable system covered with the layer of alginate gel (C, D). It has been found that neither the immersion time nor the temperature of saturation in $CaCl_2$ influences the setting time of the system, but on the other hand we have established that an increase in the concentration of cross-linker makes the time of material gelation fairly shorter (figure 2A).

Two generations of the cylinder-shaped biomaterials were tested in order to evaluate mechanical properties of the injectable system. Both dried samples and newly formed scaffolds wetted with an alginic layer were subjected to the study. It was found that the "dry" specimens tend to produce a stiffer architecture due to the dried alginic phase covering the beads' surface, which losts its gel-like elastic structure during the drying process. Young's modulus for "dry" materials reached 16.19 ± 2.35 MPa and the compressive strength amounted to 1.97 ± 0.77 MPa. In the case of the hydrated material, both elastic modulus and compressive strength obtained had lower values and reached 9.54 ± 0.62 MPa and 0.94 ± 0.07 MPa, respectively.

The results of the cytocompatiblity tests were successful since they met expectations and showed the biological properties of basic materials.

The mutagenicity test reveals that the extracts of the chitosan-based material within the concentration range tested do not increase the number of revertants

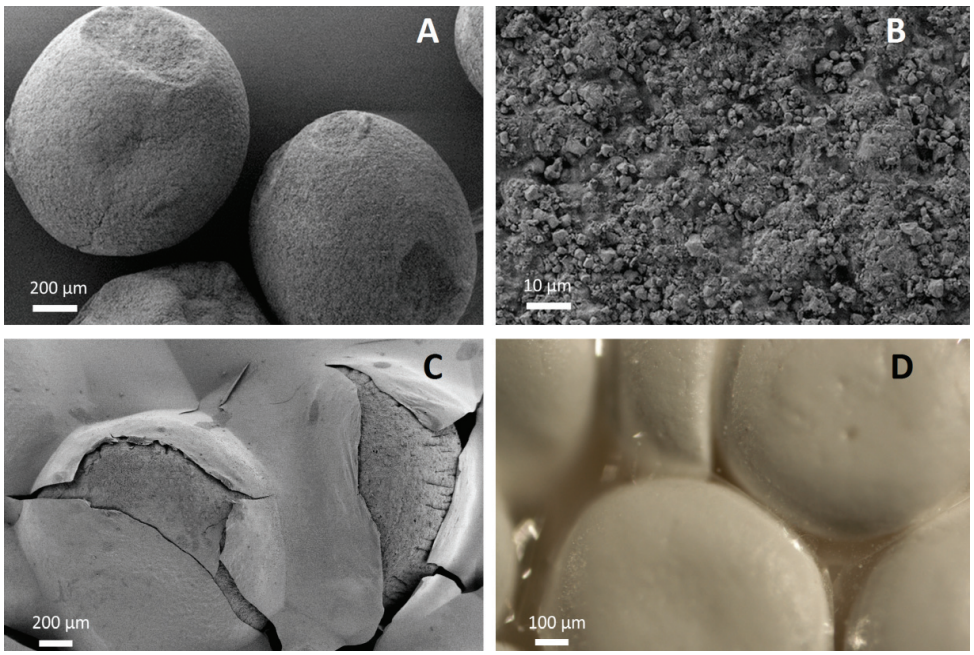

Fig. 1. Morphology and topography of CH/TCP particles obtained by SEM analysis

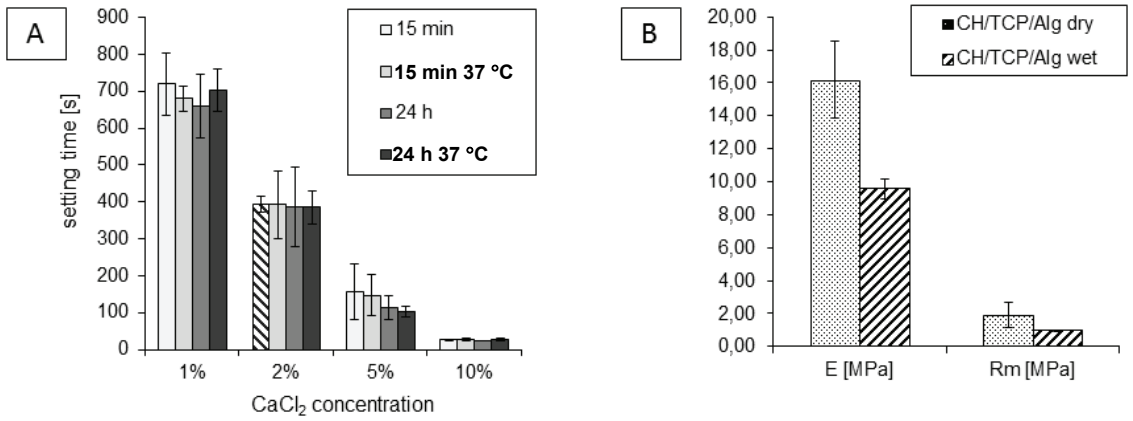

Fig. 2. Effect of calcium chloride concentrations (1, 2, 5, 10%), saturation times (15 min, 24 h) and immersion conditions (RT, 37 °C) on the setting time of injectable system (A). Mechanical properties; *E* – Young's modulus, *Rm* – compressive strength (B)

of *Salmonella typhimurium* strains, both with or without metabolic activation. The number of revertants in the concentrations tested corresponds to the number of revertants in control cultures. The results of the Ames test show that our material is not genotoxic within the range of the concentrations tested (from 0.01 mg/ml to 0.4 mg/ml). There is no relationship between the number of his^+ revertants and the raise of concentration of the substance tested. The doubling of the number of his^+ revertants compared to that of spontaneous revertants was not observed.

As was shown by the qualitative morphological evaluation of the cell cultures after 48-hr exposure to the extract of the material tested or after 24-hr direct contact with it , also confirmed in agar diffusion test, no malformed or degenerated cells were observed. The cells proved to be free of intracytoplasmatic granules. The retardation of cell growth or lysis was not noticed throughout all the culture areas in each of the tests.

The results obtained in these studies based on the European/Polish Standard PN-EN ISO 10993 prove that the material tested is neither genotoxic (mutagenicity evaluated both with or without metabolic activation) nor cytotoxic.

A local tolerance of material was tested on rabbits after epidermal and intradermal application. It has been proved that neither a solid graft applied directly to intact skin nor the saline extract injected intracutaneously produces any irritant effects. Similarly, the results of

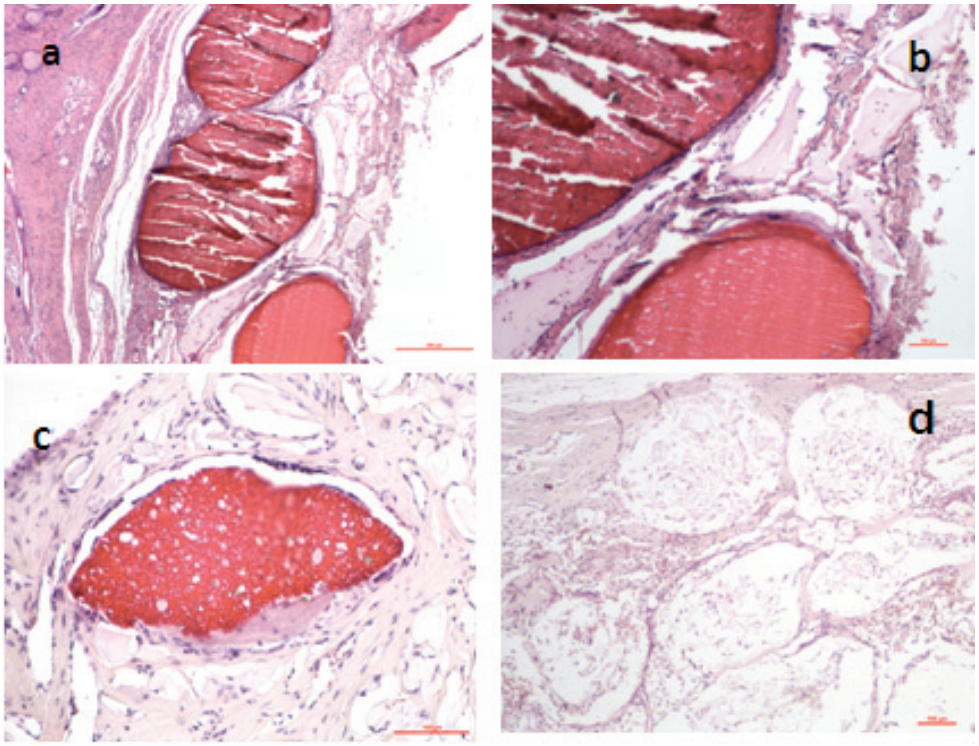

Fig. 3. Histological evaluation after 4 and 12 weeks after implantation; a, b, c – CH/TCP/Alg biomaterial, d – control; a, b – woven bone tissue around the graft observed after 4 weeks, c – new bone formation and degradation of the graft after 12 weeks, d – slight bone formation and inflammatory response around the commercially available biomaterial

delayed hypersensitivity testing on guinea pigs have shown that the graft exhibits no sensitizing potential in animals. Therefore the risk of allergic reactions in humans is practically negligible. It has also been proved that in acute systemic testing the saline extract of our biomaterial injected intravenously into mice at the maximum allowable volume is non-toxic.

Histological examination (figure 3) after one month revealed an early new bone formation observed around the biomaterial. There were no symptoms of purulent inflammation (with neutrophils infiltrate) and necrosis, nor granulomatous inflammation (with giant and epithelioid cells). There was minimal and mild chronic infiltrate composed of lymphocytes and macrophages around the biomaterial. Additionally, minimal capillary proliferation and fibrosis were observed. Microscopic examination after 3 months from the surgery revealed trabecular bone formation around our biomaterial with no significant inflammatory response.

4. Discussion

In the present work, the formation of the self-setting biomaterial obtained from chitosan, calcium phosphate and alginate salt was proposed. The biomaterial was characterized in terms of its morphology, self-setting time with respect to calcium ion concentrations and temperature of granule saturation in the cross-linker solution. Mechanical properties were analyzed for dried and wetted biomaterials. The trials confirm the possibility of manufacturing such a material with precisely definable physical and chemical properties and consistent batch quality.

Cell cultures and bacterial strains provide a convenient, controllable and repetitive tool for a preliminary evaluation of the biological response to such materials. Genotoxicity and cytotoxicity are important factors affecting the systemic compatibility of an implantable material. In general, genotoxicity testifies to the presence of a DNA-reactive component which may result in mutagenicity and carcinogenicity. In vitro cytotoxicity is a simulation of the biological response to the material by the exposition of the cell cultures to the extracts or direct contact with the material. Due to serious and life-threatening consequences these tests are gaining increasing public interest [8].

Local tolerance and systemic toxicity testing as well as the evaluation of sensitizing potential are also of a significant importance for the safety assessment of medical devices. This evaluation should always be

performed prior to human exposure to the material. The above mentioned tests prove that CH/TCP/Alg material ensures the general standard for grafting device and produces no health hazards to future patients in terms of local tolerance, allergic reactions and systemic safety.

The histological evalution of the specimens removed revealed the presence of newly formed bone tissue around the biomaterial without significant acute and chronic inflammatory response.

Guided bone regeneration (GBR) has significantly changed implant dentistry in the past 20 years. The lack of bone in the alveolar ridge presents a great challenge to densists being responsible for a successful rehabilitation of the stomathognatic system. Hard tissue augmentation is becoming more common than the usage of bone substitute materials in oral surgery.

Our project was aimed at designing an alloplastic graft for the above mentioned procedures. The material tested showed its cytocompatibility, no adverse reactions when in direct contact with living tissues and a safe use of its extracts in laboratory animals. In vivo studies on a rat model revealed the formation of bundle bone around the grafts and no signs of irritation to the surrounding tissue.

The preclinical evaluation performed on the new, alloplastic, injectable bone substitute offers the possibilities of patent pending and advanced in vivo testing.

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