e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 66 (2018), No 4
https://doi.org/10.3409/fb_66-4.17

Cytotoxicity and Proinflammatory Cytokine Expression in Response to Eluates of a Ceramic-Polymer Composite Biomaterial in Cultured Human hs-27 Cells; Possible Application for Bone Regeneration

Joanna ZARZECKA, Jerzy FRĄCZEK, Agnieszka SKÓRSKA-STANIA, Agnieszka JELONEK, Barbara CZOPIK, Paweł MYCIŃSKI, and Maria WRÓBEL✉

Accepted December 12, 2018 *Published online December 20, 2018* *Issue online December 31, 2018*

Original article

ZARZECKA J., FRĄCZEK J., SKÓRSKA-STANIA A., JELONEK A., CZOPIK B., MYCIŃSKI P., WRÓBEL M. 2018. Cytotoxicity and proinflammatory cytokine expression in response to eluates of a ceramic-polymer composite biomaterial in cultured human hs-27 cells; possible application for bone regeneration. Folia Biologica (Kraków) **66**: 159-164.

The idea of bone tissue regeneration calls for the development of new biomaterials. We designed a novel ceramic-polymer composite material that expresses the feature of drug carrier to restore facial and cranial bone defects. The ceramic phase consists of BCP with different proportions of HAp/β-TCP, while the polymer phase is poly(D, L-lactide) which is a carrier for clindamycin. The purpose of this study was to determine whether the eluates of the designed biomaterial have the potential to cause inflammatory response or express cytotoxicity *in vitro*. The elution was carried out for 24 hours or 6 days. Cells were incubated for 24 or 48 h with eluates of six types of materials: HP1 group (HAp with polylactide in composition 61%-39%); HP2 group (HAp with polylactide in composition 80%-20%); HPC group (HAp with polylactide and clindamycin); BP group (BCP with polylactide); BPC group (BCP with polylactide and clindamycin); B group (BCP). Cytotoxicity was determined with a commercial cytotoxicity kit on human fibroblasts from the hs-27 cell line. ELISA was used to measure cytokine expression for pro-inflammatory IL-6 and IL-8. Eluates of the novel ceramic-polymer composite material with the feature of a drug carrier (BCP and polylactide with clindamycin) did not produce a cytotoxic effect in the human fibroblast hs-27 cell line, nor did any of the tested biomaterials. The tested materials did not influence the production of pro-inflammatory cytokines. Therefore the novel ceramic-polylactide composite material may be further tested *in vivo* as a promising alternative for known biomaterials in bone defect reconstruction.

Key words: Cytotoxicity, hs-27 cell line, hydroxyapatite, polylactide, pro-inflammatory cytokines, β-tricalcium phosphate.

Joanna ZARZECKA, Barbara CZOPIK, Paweł MYCIŃSKI, Department of Conservative Dentistry with Endodontics, Jagiellonian University, Collegium Medicum, Montelupich 4, Kraków, Poland.
Jerzy FRĄCZEK, Maria WRÓBEL✉, Department of Medical Biochemistry, Jagiellonian University, Collegium Medicum, Kopernika 7, Kraków, Poland.
E-mail: mbwrobel@cyf-kr.edu.pl
Agnieszka SKÓRSKA-STANIA, Agnieszka JELONEK, Department of Crystal Chemistry and Crystal Physics, Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, Kraków, Poland.

The idea of regenerative dentistry calls for the development of new biomaterials and biological methods of treatment. This biological approach would allow for the regeneration of every tissue of the maxillofacial region that was injured or lost due to disease or trauma. A model bone-replacing material should fulfill defined criteria: it should be non-toxic, noncaustic and biocompatible. This material should express bioactivity, biocompatibility and osteoconductivity. Once implanted *in vivo* it should be overgrown with newly synthesized autologous bone and has to be completely replaced by this tissue with time.

Two of the most commonly used bone-replacement materials: hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) are very similar to the mineral phase of human teeth and bone. HAp is highly biocompatible, expresses osteoconductivity, is bioactive and has the potential to form chemical bonds with human bone tissue. β-TCP is bioactive, as well as biocompatible and resorbable (ŚLÓSARCZYK 2003; KOEPP *et al*. 2004; SOPYAN *et al*. 2007). However, both of these materials express some undesirable features. HAp is not degraded *in vivo*, and therefore is not fully replaced by bone tissue, being only a type of construct that supports bone synthesis on its surface, while β-TCP expresses uncontrolled process of resorption, which implies differences between the amounts of newly synthetized bone tissue and implant material (NERY *et al*. 1992). Biphasic Calcium Phosphate Ceramic (BCP) is a mixture of HAp and β-TCP that possesses the advantageous features of both of these materials. However, although it is chemically stable in a biological environment, it has a high value of Young Module and therefore can crack once implanted *in vivo* (LEGEROS *et al*. 2003).

Not only bioceramic materials are used as bone-replacing implants. Natural or synthetic polymers, such as polylactic acids (PLLA or PDLLA) are hydrolyzed *in vivo* to lactic acid which is a natural component of the human body. They are biocompatible, express good mechanical resistance and can be easily shaped into polylactide implants of different sizes and forms (LI CHUN *et al*. 2008). PLLA and PDLLA can also be used as drug carriers, which is subject to experimentation in the field of biomaterial research. The use of drug carries decreases drug toxicity, as the therapeutic substance is implanted directly into the place of disease and only there does it express its biological action (COSIJNS *et al.* 2007; FERRAZ *et al.* 2007; HONG *et al.* 2011; KANELLAKOPOULOU and GIAMARELLOS-BOURBOULIS 2000; MANGANO *et al.* 2003). However, the chemical resistance in the *in vivo* environment of polymers is decreased when compared with ceramic materials, BCP on the other hand is not elastic and therefore susceptible to cracking. In this study we used hydroxyapatite with polylactide in two different concentrations as one of the tested materials.

In order to eliminate the disadvantages and increase the number of beneficial features in one material that can be used to restore facial and cranial bone defects, a novel ceramic-polymer composite material has been obtained from BCP and polymers, which has the feature of a drug carrier. Its ceramic phase consists of BCP with different proportions of HAp/β-TCP, while the polymer phase is poly(D, L-lactide) that is a carrier for clindamycin. The structure of the material is organized into porous granules of 250 μm to 1000 μm size which should enable a better placement inside bone defect and decrease the risk of material movement after implantation, diminishing the risk of post-operative complications. The porous structure creates an ideal niche for the newly synthesized bone to grow which should result in higher bond strength between the material and bone tissue. The polylactide layer improves not only the elasticity of the material, but also the adhesion of cells to the surface of the implanted material. The possibility of drug deposition inside the material makes it possible to treat the inflammatory response after surgical procedure and eliminate post-operative complications.

Taking into consideration all advantages and disadvantages of the mentioned types of bone-replacing materials, we created four different experimental groups of known materials in different combinations of compounds: HAp with polylactide in composition 61%-39%; HAp with polylactide in composition 80%-20%; HAp with polylactide and clindamycin; BCP, and compared them with the novel ceramic-polylactide composite material with a feature of drug carrier – BCP + polylactide with or without clindamycin invented by our research group.

One of the steps in the evaluation of usefulness of any medical product or material is to check their possible cytotoxic effect on human cells. This can be tested either by directly using the material in tissue cultures or by preparing eluates of the materials in different solvents followed by the evaluation of the eluate cytotoxicity in cell culture conditions (ISO 10993 standard "Biological evaluation of the medical devices").

The purpose of this study was to determine whether the ceramic-polylactide composite material with a feature of drug carrier – BCP + polylactide with or without clindamycin (eluates used in experimental groups 4 and 5) – has the potential to cause inflammatory response or expresses cytotoxicity *in vitro* and to compare it with alternative graft materials: hydroxyapatite with polylactide in two different concentrations (group 1 and 2), as well as hydroxyapatite with polylactide and clindamycin (group 3) and single BCP (group 6).

Materials and Methods

Abbreviations
BCP – biphasic Calcium Phosphate Ceramic
HAp – hydroxyapatite
PDLLA – poly(D, L-lactide)
PLLA – poly(L-lactic acid)
β-TCP – β-tricalcium phosphate

Preparation of the eluates from the implant materials

To test the influence of the implant materials in *in vitro* conditions, eluates of the prepared materials were divided into 6 experimental groups after dissolving in polar and non-polar solvents. The 6 experimental groups were: group 1 – HP1 (HAp with polylactide in composition 61%-39%); group 2 – HP2 (HAp with polylactide in composition 80%-20%); group 3 – HPC (HAp with polylactide and clindamycin); group 4 – BP (BCP with polylactide); group 5 – BPC (BCP with polylactide and clindamycin); group 6 – B (BCP). All materials from the 6 groups were prepared in the laboratory. Pure DMEM medium was chosen as a polar solvent and DMSO as a polar aprotic solvent for non-polar compounds. 20 mg of each implant material was used for elution in 100 µl of each solvent according to recommendations of the ISO regulation; elution was carried out in 37°C for 24 hours or 6 days for a prolonged time of material release. The eluates were prepared according to the ISO 10993 standard "Biological evaluation of the medical devices" (ISO 10993 standards). We used only one selected amount of eluates to treat cells, because DMSO concentration in the medium was already about 1% and there was no possibility to use multiple samples, they could be used in the case of dissolution in DMEM only. Because the highest obtained concentrations did not show cytotoxic activity, dilutions were not prepared.

Cell culture conditions for the evaluation of possible cytotoxicity of the eluates from implant materials

Human fibroblasts from the hs-27 cell line were cultured in the cell culture medium DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone), penicillin (1000 U/ml) (Gibco) and streptomycin (1000 U/ml) (Gibco) – a complete cell culture medium. We decided human fibroblasts will be good model cells for cytoxicity evaluation in this study, as they are widely propagated in human body, especially in connective tissue, are relatively easy in laboratory procedures such as culture, cell passages and others. To test the cytotoxicity of the eluates from implant materials in cell culture conditions, fibroblasts were seeded on a 96-well tissue culture plate (BD Falcon) in the amount of $8x10^3$ cells per well for 24 hours before the experiment. On the day of the experiment, the culture medium was replaced with a fresh one and the eluates were added in the amount of 2 µl per 200 µl of the medium in the well. Cells were incubated with the eluates or as controls for the next 24 or 48 hours as indicated and then cell culture supernatants were collected to measure the possible cytotoxicity effect on the cells.

Cytotoxicity was determined using the Cytotoxicity Detection KitPLUS (LDH) (Roche) according to manufacturer's instruction. As a negative control, appropriate amounts of either pure DMEM or DMSO were added at the beginning of the experiment and for the positive control of the cytotoxic effect cells were additionally treated with 0.1% Triton X-100 (Sigma) or appropriate buffer from the Cytotoxicity Detection KitPLUS (LDH) (Roche) for 2 hours before collecting cell culture supernatants to ensure their complete lysis. The product of the reaction from the Cytotoxicity Detection KitPLUS (LDH) was measured using a spectrophotometer microplate reader (Epoch, BioTek) with the software Gen5 2.0. Experiments were performed four times in triplicates on each plate, which were averaged before entering the analysis.

Measurement of cytokine production by eluates from implant materials

To detect the production of cytokines in cell cultures upon treatment with the material eluates, cells were seeded a day before the experiment on 24-well plates in the amount of $60x10^3$ cells per well and cultured in 0.8 ml of complete medium in the same cell culture conditions as described above. On the day of the experiment the medium was replaced with 0.8 ml of fresh one in each well and the appropriate eluates were added in the amount of 1 µl per each 100 µl of medium in a well. Additionally, the LPS was used as a positive control of cytokine production in a concentration of 400 ng/ml and pure DMEM or DMSO were added in amounts similar to eluates as negative controls. Cells were incubated with appropriate factors for 24 hours and then cell culture supernatants were collected for measurement of cytokine production. The measurement of cytokine expression was performed using Quantikine ELISA 1 kits (R&D Systems) for pro-inflammatory IL-6 and IL-8 according to the manufacturer's instructions. The experiments were performed three times independently in triplicates, which were averaged before entering the analysis.

Data presentation

All data are presented in graphs as the mean ±SD. We decided that 4 and 3 independent measurements per group are enough to reach the desired precision, as it is generally accepted to present results being the average of at least three independent experiments. To achieve measurement inde-

pendence, each experiment started with a new cell bank with a comparable number of passages.

Statistical analysis was not carried out because of the number of results (9 to 12 for cytotoxicity determination and 6 to 9 for the production of cytokines) and sometimes large standard deviations. Since the difference between the positive control and the tested samples was undisputed, statistical analysis would not bring any additional information.

Results and Discussion

The results indicate that none of the evaluated eluates from any experimental group induced a cytotoxic effect in the human fibroblast hs-27 cell line. Material eluates with either DMEM or DMSO did not cause a decrease in cell viability as measured with the test (Fig. 1a, b). A prolonged elution time also did not influence the cytotoxicity of the eluates (Fig. 1c, d). In the case of the negative control (pure DMEM or DMSO) and positive control (0.1% Triton), the registered cytotoxicity was 0% and 100%, respectively.

These results were additionally confirmed by microscopic analysis of cell cultures in which none of the cells of the experimental groups differed from the negative control group and all showed signs of healthy morphology and growth (data not shown). In conclusion, the novel ceramic-polymer material fulfills the criteria of a biomaterial in the aspect of being non-cytotoxic.

Since implant materials can interact with living tissues of the organism, another question was asked: if they can induce an inflammatory response by initiation of the production of inflammatory cytokines. There are many tests available to check material cytotoxicity in laboratory conditions such as: MTT assay, agar diffusion test, filter diffusion test and many more. In this study we investigated whether the eluates of the implant materials can impact the production of pro-inflammatory cytokines using IL-6 and IL-8 Cytotoxicity Detection KitPLUS (LDH) (Roche). The results indicate that the eluates tested here do not

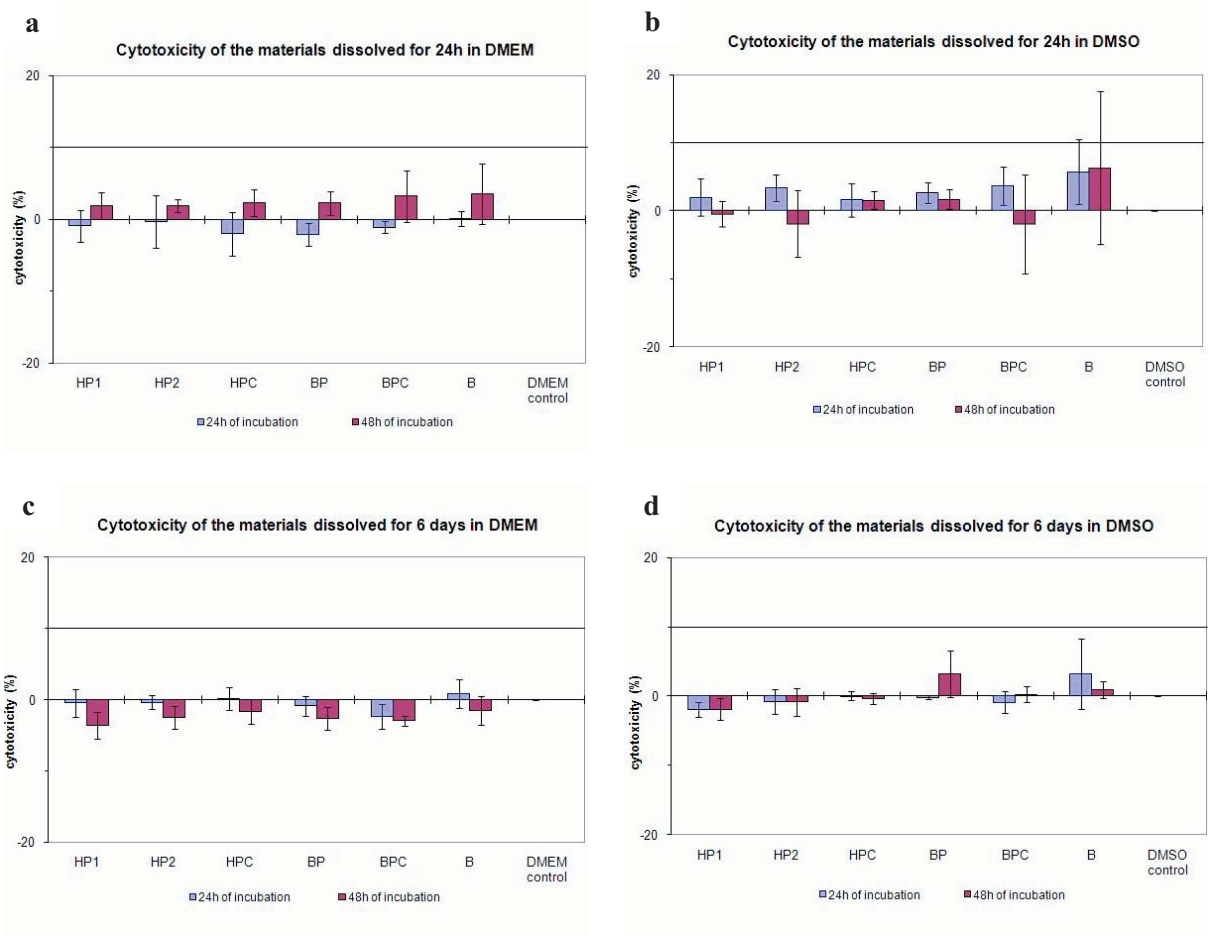

Fig. 1. Cytotoxicity of the implant materials (group 1 to 6) dissolved for 24 h or 6 days in DMEM (a) (c) or DMSO (b) (d). Positive control – 0.1% Triton X-100 (Sigma) or appropriate buffer from the Cytotoxicity Detection KitPLUS (LDH) yielded 100% cytotoxicity (result not shown in the graph to avoid its overloading). Negative control – DMEM or DMSO solutions. HP1 – HAp with polylactide in composition 61%-39%; HP2 – HAp with polylactide in composition 80%-20%; HPC – HAp with polylactide and clindamycin; BP – BCP with polylactide; BPC – BCP with polylactide and clindamycin; B – BCP. Bars represent mean ±SD of 4 independent experiments.

induce the production of neither IL-6 nor IL-8 – strong pro-inflammatory cytokines (Fig. 2a-d).

However, it should be noted that these data indicate only the fact that the eluates of implant materials do not influence the production of pro-inflammatory cytokines IL-6 and IL-8, and we can only suspect that the implanted material should behave similarly. These results should be further confirmed in *in vivo* studies, where novel ceramic-polymer material will be implanted into tissue at a macroscopic scale.

One of the most interesting and rapidly developing areas for biomaterials applications is the controlled and targeted delivery of drugs. We managed to modify novel ceramic-polymer material to be a drug carrier for one of the most widely applied antibiotics in dentistry – clindamycin. We proved that the addition of clindamycin (group 5) does not cause any changes in cytotoxicity or potential to cause inflammatory response in comparison to ceramic-polymer material without the drug (group 4). The concentration of clindamycin and the possible amounts of clindamycin released to the eluates are not described in the current study, as we aimed at only determining if clindamycin presence in the biomaterial will have any influence on inflammation in cell culture. In case of the induction of an inflammatory response or any other adverse effects on the cells, no further experiments would be carried out. The concentration of clindamycin needed for release of a particular amount will be the subject of our next experiment.

The obtained results are similar to those in other studies on biocompatibility of this type of biomaterial. LEI *et al.* (2012) studied properties of biphasic calcium phosphate scaffolds coated with HAp/PLLA composites for bone tissue engineering applications. *In vitro* cytotoxicity was investigated with the MTT assay on the rat osteoblastic ROS 17/2.8 cell line. The results showed good biocompatibility, showing no negative effect on cell growth and proliferation. We therefore decided to use the human fibroblast cell line in our study, as more relevant according to possible future applications, as well as the Cytotoxicity Detection Kit-

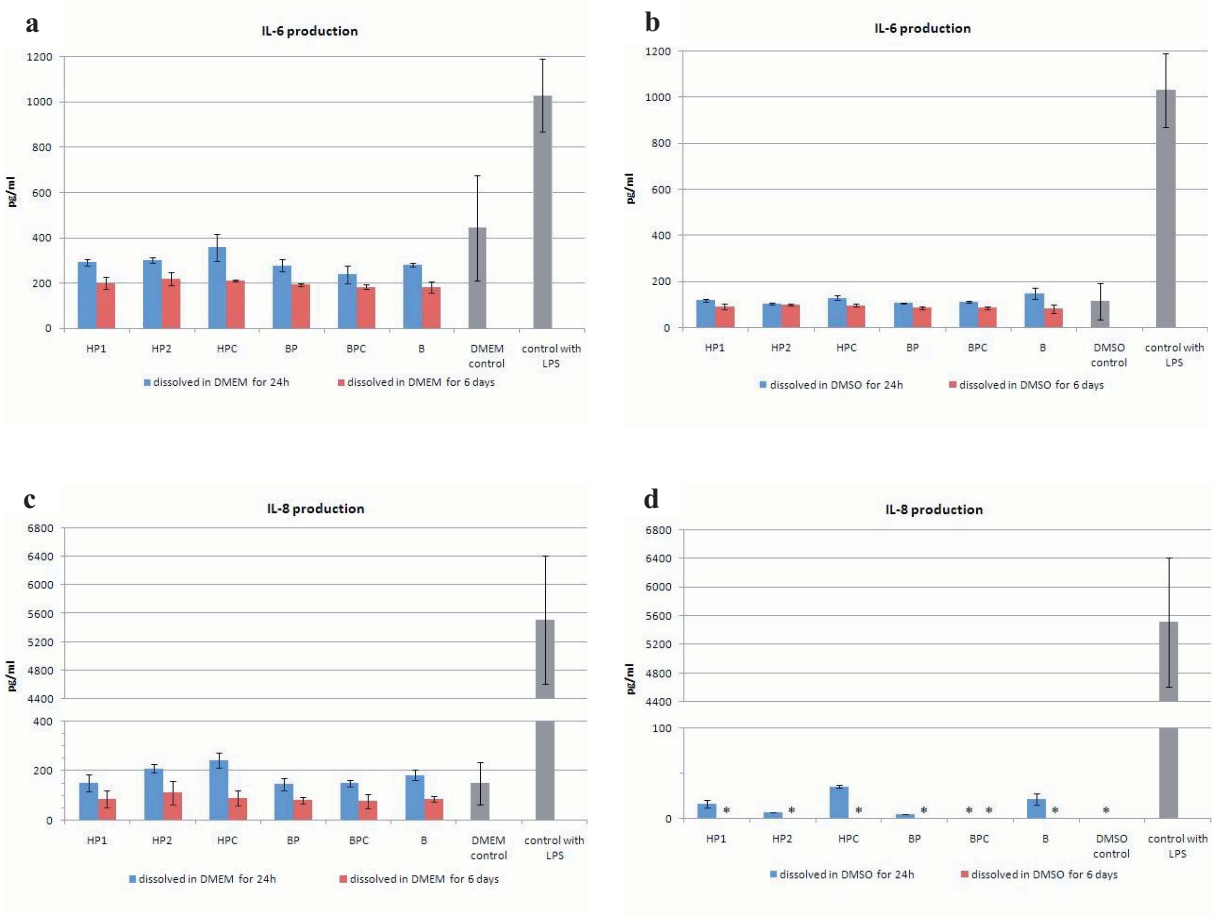

Fig. 2. IL-6 (a, b) and IL-8 (c, d) production by human fibroblasts hs-27 treated with the implant material eluates (group 1 to 6) dissolved for 24 h or 6 days in DMEM or DMSO. LPS (400 ng/ml) was used as a positive control of cytokine production and pure DMEM or DMSO solutions in amounts similar to eluates as negative control. HP1 – HAp with polylactide in composition 61%-39%; HP2 – HAp with polylactide in composition 80%-20%; HPC – HAp with polylactide and clindamycin; BP – BCP with polylactide; BPC – BCP with polylactide and clindamycin; B – BCP. Bars represent mean ±SD of 3 independent experiments; * – not detected.

PLUS (LDH), to confirm lack of cytotoxicity with a different method.

Eluates of a novel ceramic-polylactide composite implant material synthesized by us (BCP and polylactide with clindamycin) do not cause cytotoxicity in human fibroblasts, nor the production of pro-inflammatory cytokines *in vitro*. Future tests should include its biocompatibility in respect to chemical composition, to avoid adverse tissue reactions after implantation, as well as its resistance to biological degradation, strength to sustain loading and ability to minimize bone resorption.

Further *in vivo* studies should determine if it is a potentially good alternative for known biomaterials in bone defects reconstruction and could be a useful tool to replace or augment the function of human bone tissue in the future.

Acknowledgements

The study was financed with subvention no K/ZDS/003966/2014-2015.

Author Contributions

Research concept and design: J.Z., A.S.-S., A.J., P.M., M.W.; Collection and/or assembly of data: J.F., A.S.-S., A.J.; Data analysis and interpretation: J.F., M.W.; Writing the article: J.F.; Critical revision of the article: J.Z., B.C., P.M., M.W.; Final approval of article: J.Z., P.M.

Conflict of Interest

The authors declare no conflict of interest.

References

COSIJNS A., VERVAET C., LUYTEN J., MULLENS S., SIEPMANN F., VAN HOOREBEKE L., MASSCHAELE B., CNUDDE V., REMON J.P. 2007. Porous hydroxyapatite tablets as carriers for low-dosed drugs. Eur. J. Pharm. Biopharm. **67**: 498-506.

FERRAZ M.P., MATEUS A.Y., SOUSA J.C., MONTEIRO F.J. 2007. Nanohydroxyapatite microspheres as delivery system for antibiotics: Release kinetics, antimicrobial activity, and interaction with osteoblasts. J. Biomed. Mater. Res. A. **81**: 994-1004. https://doi.org/10.1002/jbm.a.31151

HONG M.H., SON J.S., KIM K.M., HAN M., OH D.S., LEE Y.K. 2011. Drug-loaded porous spherical hydroxyapatite granules for bone regeneration. J. Mater. Sci. Mater. Med. **22**: 349-355.

ISO 10993 standards "Biological evaluation of the medical devices". 2009. https://www.iso.org/obp/ui/#iso:std:iso:10993:-5:ed-3:v1:en

KANELLAKOPOULOU K., GIAMARELLOS-BOURBOULIS E.J. 2000. Carrier systems for the local delivery of antibiotics in bone infections. Drugs **59**: 1223-1232.

KOEPP H.E., SCHORLEMMER S., KESSLER S., BRENNER R.E., CLAES L., GÜNTHER K-P., IGNATIUS A.A. 2004. Biocompatibility and osseointegration of beta-TCP: histomorphological and biomechanical studies in a weight-bearing sheep model. J. Biomed. Mater. Res. B. **70**: 209-17. https://doi.org/10.1002/jbm.b.30034

LEGEROS R.Z., LIN S., ROHANIZADEH R., MIJARES D., LEGEROS J.P. 2003. Biphasic calcium phosphate bioceramics: Preparation, properties and applications. J. Mater. Sci. Mater. Med. **14**: 201-209.

LEI N., JINPING S., PENG Z., SHUIBIN F. 2012 Preparation and properties of Biphasic Calcium Phosphate Scaffolds multiply coated with HA/PLLA Nanocomposites for Bone Tissue Engineering Applications. J. Nanomater. 1-11. http://dx.doi.org/10.1155/2012/213549

LI CHUN LIN, SHWU JEN CHANG, SHYH MING KUO, GREGORY CHENG-CHIE NIU, HAO KAI KENG, PEI HUA TSAI 2008. Preparation and Evaluation of β-TCP/Polylactide Microspheres as Osteogenesis Materials. J. Appl. Polym. Sci. **108**: 3210-3217. https://doi.org/10.1002/app.27309

MANGANO C., BARTOLUCCI E.G., MAZZOCCO C. 2003. A new porous hydroxyapatite for promotion of bone regeneration in maxillary sinus augmentation: clinical and histologic study in humans. Int. J. Oral. Maxillofac. Implant. **18**: 23-30.

NERY E.B., LEGEROS R.Z., LYNCH K.L., LEE K. 1992. Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/beta TCP in periodontal osseous defects. J. Periodontol. **63**: 729-735. https://doi.org/10.1902/jop.1992.63.9.729

SOPYAN I., MEL M., RAMESH S., KHALID K.A. 2007. Porous hydroxyapatite for artificial bone applications. Sci. Technol. Adv. Mater. **8**: 116-23. https://doi.org/10.1016/j.stam.2006.11.017

ŚLÓSARCZYK A. 2003. Biomateriały ceramiczne Ceramic Biomaterials). In: Biomateriały (Biomaterials). Vol. 4. S. BŁAŻEWICZ, L. STOCH ed. Akademicka Oficyna Wydawnicza EXIT, Warszawa): 99-149. (In Polish).