Acta Veterinaria (Beograd), Vol. 56, No. 5-6, 541-551, 2006.

DOI: 10.2298/AVB0606541M UDK 619:617.3

BIOCOMPATIBILITY OF NANOSTRUCTURED CARBONATED CALCIUM HYDROXYAPATITE OBTAINED BY HYDROTHERMAL METHOD

MARKOVIĆ D*, ŽIVOJINOVIĆ VESNA*, JOKANOVIĆ V** and KRSTIĆ V***

**Clinic of Preventive and Pediatric Dentistry, School of Dentistry, Belgrade;*
***Institute of Nuclear Sciences "Vinča", Beograd; ***Department of equines, small animals, poultry and wild diseases, Faculty of Veterinary Medicine, Belgrade*

(Received 31. March 2006)

Evaluation of biomaterials as safe and effective therapies needs preclinical models to estimate their biologic potential. This paper investigates biocompatibility by the in vivo assessment of the muscle tissue reaction after implantation of the hidrothermally produced calciumhydroxyapatite. Specific attention has to be given to the synthesis technique which influences the stereology of the material and the behaviour of the material in living tissues. The synthesized powders of hydroxyapatite are preferentially carbonated hydroxyapatite of the B type in the form of agglomerates that accommodate two-modal size pores of 1.5-10 nm and 50-200 nm. The particles are built from crystallites of 8-22 nm in size, bind inside of the prime particles sized is between 10 and 63 nm. They form agglomerates of 200 nm in size and these were further clustered building up the biggest agglomerates of 5-20 μm. Biocompatibility assessment revealed that only mild to moderate inflammatory reaction was seen around the calciumhydroxyapatite implants. Calciumhydroxyapatite failed to show any substantional toxicity.

Key words: biocompatibility, carbonated nano-structured hydroxyapatite, hydrothermal synthesis, tissue implantation

INTRODUCTION

Calcium hydroxyapatite (CHA) is a superior biocompatible material because it contains Ca^{2+}, $P0_4^{3-}$ and OH^- ions; the ions that human's tissues and bones have in relatively high percentage (Hench, 1991; Hench, 1998; Le Geros and Le Geros, 1998). In the form of very fine crystals CHA accounts for approx 58 per cent of bone mass in mammals. Together with the biocompatibility and non-toxicity it demonstrates excellent osteoconductivity, (Boskey and Posner, 1976; Zhang and Gonsalves, 1998; Hench, 1998; Katz and Harper, 1986; Hienerauer *et al*., 1991; Zambonin and Grano, 1995) and is widely used for bone reparation and orthopedic replacements, as well as for dental replacements and reparations (Panda *et al*., 2003; Jokanovic and Uskokovic, 2005).

An interesting approach research is related to hydroxyapatite with a higher chemical and biochemical activity on the interface with living tissues (i.e proteins of living organisms) enabling in that way a suitable binding to them.

Hydroxyapatite ceramic is also used as a protective coating of dental implants, in the bulk form as bone replacement of low (type B) or high (type F) porosity, and in the granular form for filling bone defects (Katz and Harper, 1986; Hienerauer *et al*., 1991; Zambonin and Grano, 1995; Panda *et al*., 2003). The commonly released ions from the hydroxyapatite ceramic are Ca^{2+} and PO_4^{3-} ions that are normally present in the tissue's fluid and therefore there is no allergic reaction of the tissue to the hydroxyapatite ceramic. In addition, the mitigative characteristic of hydroxyapatite enables proliferation of patron-ageing living - cells on the hydroxyapatite ceramic/living tissue interface facilitating the creation of new bones through the process of osteogenesis. Evaluation of biomaterials as safe and effective therapies needs preclinical models in order to estimate their biological potential.

In this paper, special attention was payed to the evaluation of biocompatibility, which was carried out by the *in vivo* assessment of the muscle tissue reaction after implantation of calciumhydroxyapatite.

MATERIALS AND METHODS

Powder synthesis and characterization

Shells of chicken eggs calcined at 900 °C, until carbon was removed completely and $CaCO_3$ dissociated to CaO, and pure $(NH_4)_2HPO_4$ (Merck p.a.) were the precursors for CHA synthesis. The solution mixture was prepared hydrothermally by using the following conditions: pressure $5x10^5$ Pa, temperature 150 °C and time of autoclaving 8h. An X-ray diffraction (XRD) method, (Philips PW 1050), with Cu-$K\alpha_{1-2}$ radiation, was used for phase analysis of CHA and determination of the crystallite size and lattice parameters. The PERKIN ELMER 983G IR spectrometer, with the KBr pastille was used for additional phase CHA powder characterization. The analysis was done in the range of 4000 cm^{-1} to 400 cm^{-1}. A scanning electron microscope (SEM JEOL 5300) has been used to analyze the morphology, size distribution and the average size of the CHA particles at the level of theirs largest agglomerates, as well as their constituents – the smallest agglomerates of hydroxyapatite; 200 nm in size. An energy dispersive spectrometer (QX 2000 – Oxford Instruments) combined with the SEM and a multichannel analyzer was used for the estimation of chemical homogeneity of synthesized CHA. The nitrogen gas absorption BET method (Sorptomatic 1990, Termoquest CE Instruments) was used for the determination of the specific surface of CHA powders. The samples for absorption measurements, weighted between 0.20 – 0.22 g, were thoroughly degassed at 150 °C for 3 hours.

Biological evaluation

Biological evaluation of hydrothermally synthesized calciumhydroxyapatite (sample 7) was performed using the *in vivo* tissue reactions in an intramuscular

implantation model on rabbits (ISO 10993-6). The sample preparation was performed under sterile conditions in an aseptic chamber. Cylindrical specimens 3 mm wide and 20 mm long were used. One part of the sample, with a length of 10 mm was coated with nonirritable sterile plastic and served as a negative control. The other part was the tested material in an active contact with the tissue (Fig 1a). Before the implantation procedure all samples were stored in sterile saline solution for 60 minutes.

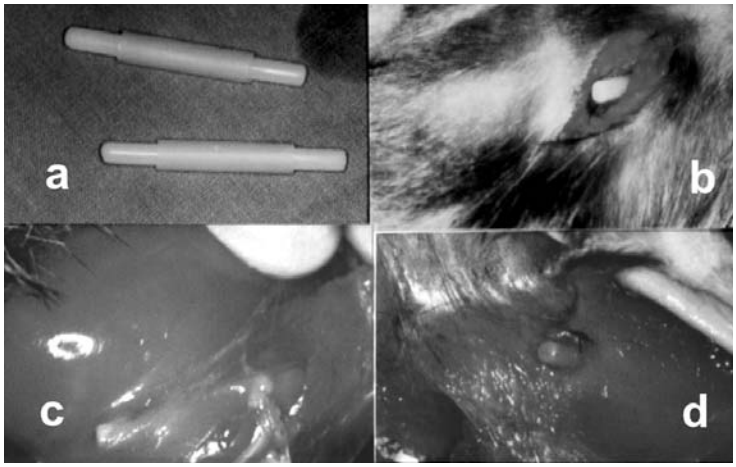

Figure 1. Implantation samples in the muscle tissue

The study animals were four, adult (nine months old) rabbits, each weighing 3-4 kg of both sexes. The animals were kept together in a facility for experimental animals with a controlled environment and were fed a standard laboratory diet. Approval was obtained from the Ethics committee of the Faculty of Stomatology, University of Belgrade (Clearance N^{o} 509/03). General anesthesia was induced by intramuscular injection of 2% xylasine (cp-Pharma, S&M) 3 mg/kg and an intramuscular injection of 5% ketamine-hydrochloride (Ralatek®, Hemofarm, Vrsac, S&M) 50 mg/kg body weight. Surgery was carried out under aseptic conditions and in a manner which minimizes trauma at the implant site. The skin of the implantation area was shaved and disinfected with an iodine solution. A full thickness flap was raised to expose the thigh muscle and samples were implanted into the muscle (Fig 1b). The skin flaps were relocated to fully cover the experimental site and were fixed with Vicryl sutures (Ethicon®, Norderstedt, Germany). The animals were held in single cages for 4 weeks, until the end of the experiment. The experimental animals were sacrificed after the evaluation period of 4 weeks by intravenous injection of a solution of pentobarbital (100 mg/L). Macroscopic assessment included examination of each implant site to record the nature and extent of any tissue reaction observed (Fig 1c, d). The muscle tissue was then prepared with the standard procedure for histological analysis. After immersion in 10% buffered formalin for 72 hours, specimens were processed into

wax. Tissue samples were grounded to a thickness of 5-7 μm and stained with haematoxylin and eosin ready for light microscopy evaluation.

Evaluation was based on the following biological response parameters:

1. Extent of fibrosis and inflammation (0-none;1-mild;2-moderate;3-severe)

2. Degeneration as determined by changes in tissue morphology (0-none; 1-mild; 2-moderate; 3-severe)

3. Number and distribution as a function of distance from the material/tissue interface of the inflammatory cell types (polymorphonuclear leucocytes, lymphocytes, plasma cells, eosinophils, macrophages and multinucleated cells) (0-none; 1-small amount immediately by implant; 2-small number in a wider diameter; 3-large number immediately and at distance from the implant)

4. Presence of necrosis as determined by nuclear debris and/or capillary wall breakdown (0-none; 1-degenerative changes on blood vessels;2- localized necrotic changes with or without changes on blood vessels adjacent to the implant; 3-abundant (large) necrotic changes)

5. other parameters such as material debris, fatty infiltration, granuloma.

The results report included a comparative evaluation of the biological responses to tested and control materials and a descriptive narrative of the biological response.

RESULTS AND DISCUSSION

Phase analysis and microstructure characteristics of CHA powder

The obtained values for lattice parameters of hydrothermally obtained hydroxyapatite, evaluated by Reitveld's methods were a = 0.9428 nm, c = 0.6877 nm and average crystallite size diameter was 16.5 nm. Using IR spectroscopy it is found that this hydroxyapatite belongs to the carbonated B type hydroxyapatite. The explanation of this statement can be found in Jokanovic *et al*., 2006.

Analysis of SEM microphotographs in Fig. 2 shows the typical shape and size of particles hydrothermally obtained CHA powders. Powders are constituted from agglomerates which shapes are nearly the same. The size of agglomerate is between 5 and 20 μm, and they are built up from fine particles 200 nm in size. The shapes of agglomerates are irregular with oval edges due to the spherical shape of individual particles. The greatest part of the agglomerates is 10-20 μm in size. However, agglomerates as big as 40 μm and as small as 5 μm can be found as well.

Measurments taken for a more precise value for crystallite size obtained by X-ray diffraction analysis, illustrate that the microstructure of hydro-thermally synthesized hydroxyapatite has been developed throughout several stages, originating from their crystallite structure in size of 16.5 nm to the fine sub-agglomerate particles in size up to 200 nm. Smaller agglomerates, most frequently, cluster further into agglomerates of 5-20 μm in size. The smallest particle size confirmed by analysis of specific surface measurements of precipitated hydroxyapatite powders by BET absorption method is approximately

30 nm. By comparing values for particle size and crystallite size of hydroxyapatite it seems that the crystallites are indeed the finest substructure elements in hydroxyapatite particles. Similarly to the size distribution at various hierarchical levels, the pore distribution among them was also estimated. It is obviously, multimodal and follows up not only the size of primary particles – the smallest ones – but also the size of other particles packed into powder clustered particles. The pores are distributed from the smallest ones, in the range of 1.5-10 nm, up to the largest in the range of 50-200 nm. The largest pores correspond to largest particles, approximately 200 nm in size, clustered most frequently into agglomerate particles 20 μm big, what can be seen on the SEM microphotographs of synthesized CHA.

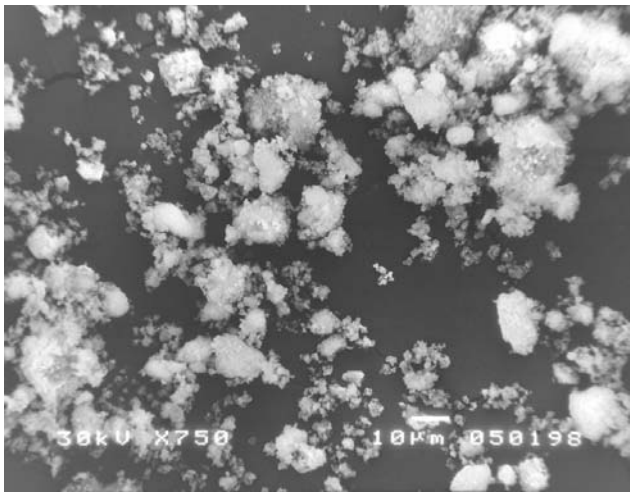

Figure 2. Characteristic microstructures of hydrothermally obtained hydroxyapatite

Biological evaluation

Biocompatibility of dental materials is evaluated on the basis of biological effect assessment by *in vitro* tests, by animal experimentations and by clinical studies on humans (Mjor, 1980; Mendes *et al*., 2001; Markovic *et al*., 2004). Cytotoxicity assay, using established laboratory cell lines according to the ISO methodology, as a first approach in a standard assay for toxicity screening of dental materials exhibited good biological acceptance (response) for samples of calciumhydroxyapatite (Gomes *et al*., 2001). Those results were an introduction for further biocompatibility research on experimental animals since results obtained from the cytotoxicity testing are not sufficient to obtain adequate knowledge on the biocompatibility of a biomaterial (ISO 10993-6). In order to define the define biological response animal experiments are designed according to the ISO methodology and principles of good research practice (Watari *et al*., 2004).

Tests for local effects after implantation are performed for some of the components of materials which can be released during the setting phase or the biodegradation process leading to direct or indirect contact with almost every part of the organism. Mostly biocompatibility assessment of calciumhydroxyapatite is performed on bone tissue because it is predominantly used in periodontal, maxilofacial and orthopaedic surgery (Markovic *et al*., 2004; de Rocha Baros *et al*., 2002, *Denissen et al., 1980*). Introduction of the use of calciumhydroxyapatite in dental pulp pathology, on the other side, needs research that will provide a more similar tissue reaction such as the one which results from muscle tissue implantation.

The synthetic nature of biomaterials eludes possible unwanted immune defense responses therefore when compared with the negative control the true response reaction to the material can be differentiated from the simple physical presence of the foreign body.

The muscle around the test and the control implants was normal in almost all animals. The only sign of inflammation, evident after macroscopic examination of implanted sites around one sample in one animal, was *rubor* with the absence of suppuration or other acute signs of inflammation. However, histological observations at the end of the evaluation period revealed some changes. Analysis of fibrous changes in most of the CHA samples showed direct correlation of the size of irritation with the amount and type of tissue reaction, and can be compared with the control.

Table 1. Distribution of biological response parameters in experimental and control samples

Animal	Site	Biological response parameters							
		A		B		C		D	
		CHA	NC	CHA	NC	CHA	NC	CHA	NC
I	I	0	1	0	1	1	0	0	0
	II	1	1	1	1	1	0	0	1
II	I	1	1	2	0	2	0	1	0
	II	0	1	1	0	1	0	0	0
III	I	1	2	1	0	1	0	1	0
	II	1	1	0	0	0	0	0	0
IV	I	0	1	0	0	1	0	0	0
	II	1	0	1	0	0	0	1	0

Parameters: A-Extent of fibrosis/fibrous capsule and inflammation, B-Degeneration as determined by changes in tissue morphology, C-Number and distribution of the inflammatory cell types, D-Number and distribution of the inflammatory cell types, CHA –calciumhydroxyapatite, NC-negative control

CHA samples induced cystic formation encircled by connective tissue which served as a cyst wall (Figure 3, upper picture). Inflammatory infiltrate inside

of the wall was described as mild inflammation. Degenerated cells of muscle tissue with calciphic dystrophy can be seen (Figure 3, lower picture part).

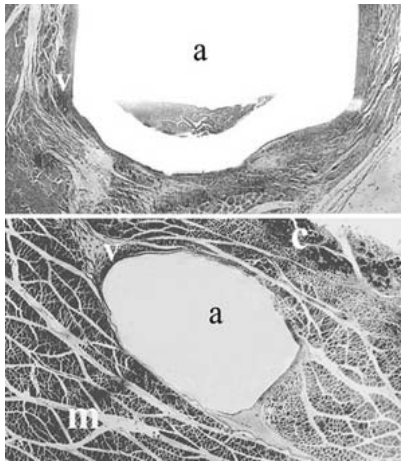

Figure 3. Cystic formation inside the muscle tissue in CHA samples (a - cystic formation, v - connective tissue, m - muscle tissue, c - calcific dystrophy (H&E, 16x))

Formation of fibrous capsules with CHA probably resulted from mechanical irritation and was not an inflammatory reaction caused by the implanted samples. Further analysis of the cyst wall showed predominantly spindle-shaped cells, young fibroblasts and fibrocytes proving the aseptic reaction caused by the implant was without signs of infection and/or allergic reaction.

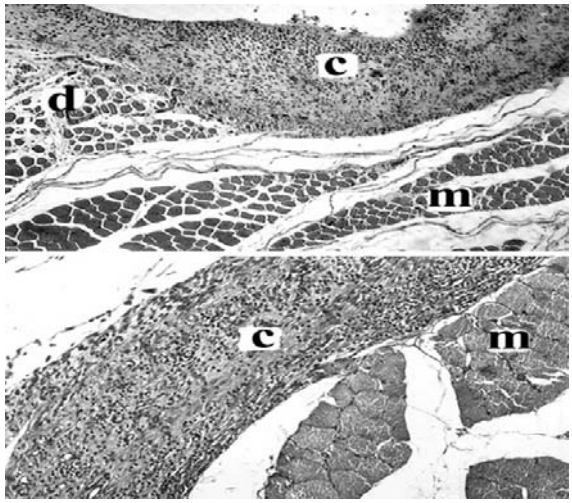

Figure 4. Cyst wall inside the muscle tissue at CHA site (c - part of cyst wall, m - muscle tissue, d - muscle cells with calcium dystrophy (H&E; 64 X))

In some CHA samples there were recorded muscle fibers which were next to the granulation tissue and showed signs of degeneration. Muscle cells with calcium dystrophy were seen in different phases of development (Figure 4). The presence of calcium dystrophy inside the muscle tissue can be caused by mechanical irritation. This is supported by fibrocytes infiltration originating probably from muscle bundles covering. Atrophic changes in muscle tissue morphology with CHA samples immediately around the implanted samples were mild to moderate and can be compared with the negative control (two samples) (Table 1).

Furthermore, muscle tissue had multinucleated cells with basophilic cytoplasm, and present granulation was present with signs of hyperplasia or calcium dystrophy (Figure 5).

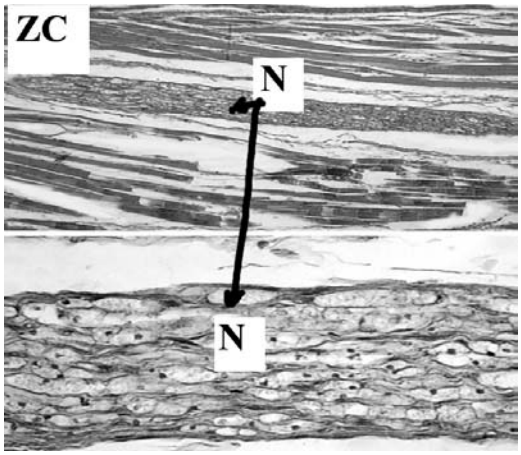

Figure 5. The wall of cystic formation in CHA sample: upper picture) N - nerve fibers, zc - the wall of cystic formation (H&E; 50x). Lower picture) N - nerve cells (detail, higher magnification) (H&E; 250x)

Inside the wall of the cystic formation nerve fibers between muscle bundles were evident on night magnification. Further magnification revealed degenerative changes of nerve cells as well as hyaline drops in some of them (Figure 5).

Results from this study showed that with CHA there was a mild infiltration of the white cells strain with a decrease in their number as the distance from the implant grew. Predominant cell infiltration at CHA samples was of mononuclear type, mostly with lymphocytes and macrophages but only in a small amount adjacent to the implant (Table 1). In only one specimen a small number of inflammatory cell types in a wider diameter was recorded. Chronic inflammation in the muscle tissue might result from the duration of the implantation period.

Degenerative changes of blood vessels were recorded in CHA samples. Diapedesis of white blood cells can be seen on some of the samples in the vein capillaries. The presence of a white thrombus, as evidence of circular disturbances resulting from the tested material, was noticed (Table 1).

The so obtained results with hydrothermally obtained hydroxyapatite are in agreement with other studies reporting a good biocompatibility of hydroxyapatite (Denissen *et al*., 1980, Jean *et al*., 1992). Furthermore, when implanted in muscle tissue this material induced a satisfactory tissue response.

CONCLUSION

The biocompability of hydrothermally obtained calciumhydroxyapatite has been investigated in this paper.

In the first part of the paper the structural and morphological characteristics of calciumhydroxyapatite is given. The measurments were made by using XRD analysis, IR spectroscopy, SEM microscopy and BET surface analysis. The results of X-ray diffraction analysis and IR spectroscopy shows that this apatite has a preferential structure of B-type. The investigation done by the SEM microstructure and the BET surface analysis clarify that all the obtained powders consist of agglomerated particles 5-20 μm big, built up from particles approx 200 nm big, and these are clusters of much smaller particles of 10-63 nm. These smallest – primary – particles are built up by crystallites as big as 16.5 nm.

The main part of investigation shows the relation of biocompability to the structure and morphological characteristics of nanostructured carbonated calciumhydroxyapatite obtained by using the hydrthermal method of synthesis. Test for the muscle tissue reaction after the implantation as a direct contact of the material and a living tissue gives a valuable data about the organism response. In agreement with our expectations and most of other researches everywhere done, CHA shows a good biological acceptance, which is expressed through the mild to moderate inflammatory reaction resulting only from the mechanical irritation.

ACKNOWLEDGEMENTS:
We wish to acknowledge the support of Ministry of Science and Environmental Protection of Republic of Serbia. We also thank M. Mitrić and Z. Nedić for their support related to XRD and IR analysis.

Address for correspondence:
Markovic Dejan,
Clinic of Preventive and Pediatric Dentistry,
School of Dentistry,
University of Belgrade,
Dr Subotica street 11,
11000 Belgrade, Serbia
e-mail: dejanmar@yubc.net

REFERENCES

1. *Boskey AL, Posner AS*, 1976, Formation of hydroxyapatite at low supersaturation, *J Phys Chem*, 80, 41-5.
2. *Cheary RW, Coelho AA*, 1992, Fundamental parameters approach of X-ray line-profile fitting, *J App Cryst*, 25, 109-21.
3. *Denissen HW, Degroot K, Makkes PC, VanDenhoof A, Klooper PJ*, 1980, Tissue response to dense apatite implants in rats, *J Biomed Mater Res*, 14, 713-21.

4. *de Rocha Barros VM, Salata LA, Sverzut CE, Xavier SP, van Noost R, Johnson A, Hatton PV*, 2002, *In vivo* bone tissue response to a canasite glass ceramic, *Biomaterials*, 23, 2895-900.
5. *Gomes ME, Reis RL, Cunha A.M, Blitterswijk CA, de Bruijn JD*, 2001, Cytocompatibility and response of osteoblastic-like cells to starch-based polymers: effect of several additives and processing conditions, *Biomater*, 22, 1911-17.
6. *Hienerauer G, McGee T, Kudej R*, 1991, Evaluation of a bioactive ceramic composite as a dental implant, *Ceram Bull*, 70, 1010-15.
7. *Hench LL*, 1991, Bioceramics: From the concept to the clinic, *J Amer Ceram Soc*, 74, 1487-510.
8. *Hench LL*, 1998, Bioceramics, *J Amer Ceram Soc*, 81, 1705-28.
9. *Hench LL*, 1998, Bioceramics, a clinical cuccess, *Ceram Bull*, 77, 67-74.
10. *ISO*/*DIS* 10993-5: In Biological evaluation of medical devices: Part 5: Tests for cytotoxicity: *in vitro* methods,1992, (E).
11. *ISO* 10993-6: In Biological evaluation of medical devices – Part 6: Tests for local effects after implantation, 1994; (E).
12. *Jean A, Pouzeat J, Laboux O, Marion D, Daculsi G*, 1992, Biocompatibility of various calcium phosphate materials to dental pulp. Biomaterial-Tissue Interfaces, In: Doherty PJ et al., *Advances in biomaterials*, Elsevier Science Publishers B.V., 25-30.
13. *Jokanovic V, Izvonar D, Dramicanin MD, Jokanovic B, Zivojinovic V, Markovic D, Dacic B*, 2006, Hydrothermal synthesis and nanostructure of carbonated calcium hydroxyapatite, *J Mat Sci: Mat Med*, 17, 539-46.
14. *Jokanović V, Uskoković D*, 2005, Calcium hydroxyapatite thin films on titanium substrate prepared by ultrasonic spray pyrolysis, *Mater Trans JIM*, 46, 228-35.
15. *Katz JL, Harper RA*, 1986, Calcium phosphates and apatites, In: Bever MB, editor, *Enciclopedia of Materials Sciences and Engineering*, Pergamon Press, Oxford, 475-6.
16. *Le Geros RZ, LeGeros JP*, 1998, Dense hydroxiapatite, In: Hench LL, Wilson J, editors, *An introduction to Bioceramics*, Adv Ser Ceram 1 World Scientific Publishing Co.Pte.Ltd., London, Hong Kong, Singapure, 139-80.
17. *Markovic D, Zivojinovic V, Kokovic V, Jokanovic V*, 2004, Hydroxyapatite as the root canal system filling material: a cytotoxicity testing, *Mat Sci Forum*, 453-4, 555-61.
18. *Mendes SC, Reis RL, Bovell YP, Cunha AM, van Blitterswijk CA et al,* 2001, Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery; a preliminary study, *Biomater*, 22, 2057-64.
19. *Mjor IA*, 1980, A comparison of *in vivo* and *in vitro* methods for toxicity testing of dental materials, *Int Endodont J*, 13, 139-42.
20. *Panda RN, Hsieh HF, Chung RJ, Chin TS*, 2003, FTIR, XRD, SEM and solid state NMR investigations of carbonate-containing hydroxyapatite nano-particles synthesized by hydroxide-gel technique, *J Phys Chem Solids*; 64, 193-9.
21. *Rietveld H*, 1969, A profile refinement method for nuclear and magnetic structures, *J App Cryst*, 2, 65-71.
22. *Watari F, Yokoyama A, Omori M, Hirai T, Kondo H et al,* 2004, Biocompatibility of materials and development to functionally graded implant for bio-medical application, *Comp Sci Tech*, 6, 891-900.
23. *Zhang S, Gonsalves KE*, 1998, Preparation and Characterization of Thermally Stable Nanohydroxyapatite, *J Mater Sci: Mater Med*, 8, 25-8
24. *Zambonin G, Grano M*, 1995, Biomaterials in orthopedic surgery: Effects of different hydroxyapatites and demineralised bone matrix on proliferation rate and bone matrix synthesis by human ostoblast, *Biomater*, 16, 397-402.

BIOKOMPATIBILNOST NANOSTRUKTURNOG KARBONIČNOG KALCIJUM HIDROKSIAPATITA DOBIJENOG HIDROTERMALNOM METODOM

MARKOVIĆ D, ŽIVOJINOVIĆ VESNA, JOKANOVIĆ V i KRSTIĆ V

SADRŽAJ

Evaluacija biomaterijala kao bezbednog i efikasnog materijala u terapiji zahteva prekliničke modele da bi se ocenio njihov biološki potencijal. U ovom radu je *in vivo* ocenjivana biokompatibilnost hidrotermalno sintetizovanog kalcijumhidroksiapatita metodom implantacije u mišićno tkivo životinja. Posebnu pažnju treba obratiti na tehniku sinteze jer ona utiče na stereologiju materijala i ima uticaj na ponašanje materijala u živom organizmu. Sintetizovani prah hidroksiapatita je uglavnom ugljenični B tip hidroksiapatita u formi aglomerata koji stvaraju dva modela veličine pora od 1,5-10 nm i 50-200 nm. Čestice su izgrađene od kristalita veličine od 8-22 nm, povezani unutar primarne čestice čija je veličina između 10 i 63 nm. Oni formiraju aglomerate od 200 nm veličine a oni dalje grade veće aglomerate od 5-20 μm. Ocenjivanjem biokompatibilnosti uočena je blaga do umerena inflamatorna reakcija u mišićnom tkivu oko implantiranih uzoraka. Kalcijum hidroksiapatit nije ispoljio značajniju toksičnost.