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Mechanical Properties and Biocompatibility of a Biomaterial Based on Deproteinized Hydroxyapatite and Endodentine Cement

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***Abstract* – Hydroxyapatite is used for bone reconstruction, in order to improve its mechanical properties different substances can be added. In our study new biomaterial is created from deproteinised hydroxyaptite and endodentic cement, its mechanical properties were tested. Material was implanted subcutaneous in rats, then histological and biocompatability tests were performed. Results indicate that stuff has good mechanical properties, short setting time and gradual resorption creating porosity and ability to integrate in bone.**

***Keywords* – Natural hydroxyapatite, endodontic cement, modulus of elasticity, ultimate stress, ultimate strain, biocompatibility.**

I. Introduction

Hydroxyapatite has been used in medicine and stomatology for more than thirty years – it is biocompatible, osteoconductive and has excellent chemical and biological affinity with bone tissue [1]. However, to serve as a scaffold the material it is expected to have enough mechanical strength in order to provide structural support during bone growth and remodeling [2], [3]. Unfortunately hydroxyapatite has poor mechanical characteristics, this substance is brittle and also its ultimate stress (σ^*) and ultimate strain (ε^*) differs from the parameters of natural bone. Due to this fact hydroxyapatite has limited load-bearing clinical applications. Modulus of elasticity (E) for fresh compact bone in longitudinal loading direction is within the range of 18–22 GPa, but for hydroxyapatite on average it is 2.5–2.9 GPa [4].

One of the most widespread approaches to improve properties of this material is to produce composites by inserting reinforcing phases, including strong bioceramics (ZrO_2 or Al_2O_3), flexible biopolymers (polyethylene and poly-lactide), and recently also as very advanced material used for this purpose – nanotubes [5], [6]. These materials can significantly influence strength and toughness of a substance. In our work endodontic cement which was mixed in different proportions with natural hydroxyapatite was used. Endodontic cement is composed of several mineral oxides, it contains SiO_2, K_2O, Al_2O_3, Na_2O, Fe_2O_3, SO_3, CaO, Bi_2O, also insoluble residues of KSO_4, $NaSO_4$ and crystalline silica. Endodontic cement (*Angelus Dental Products Industry S/A*) has good mechanical properties – ultimate strength in compression is 40 MPa after 24 h and 65 MPa after 21 days, according specification of the producer. Besides that, the setting time of endodontic cement is short – it solidifies within approximately ten-fifteen minutes, thus providing quick fixation of implanted material. Natural hydroxyapatite is added to this material because in longer time period it can be resorbed and instead of it ingrowth of natural bone tissue is expected. Next, it is supposed that after resorption of natural hydroxyapatite, the structure of endodontic cement crumbles into small pieces within size of 50–75 μm due to cyclical loading and consequently in longer time period is eliminated from the body by white blood cells [7].

Natural hydroxyapatite is obtained from bovine bone which is exposed to the heat treatment not exceeding temperature more than 430 °C for five hours, until protein phase is completely eliminated. Raw material is treated in as low temperature as possible because according to literature data in lower temperatures hydroxyapatite is presented in smaller crystals and consequently increases total surface area which subsequently fosters better contact with live tissue [8]. Protein elimination is very important because otherwise it can cause immunological inflammation reactions with host body. Natural hydroxyapatite is chosen due to the fact that it retains architectural microstructure of bone after deproteinization and is better resorbed by body compared to synthetic hydroxyapatite alternative [9]–[11].

Further, it is expected to achieve an optimal scaffold with adequate porosity, when hydroxyapatite phase will start to resorb. Porosity is defined as the percentage of void space in a solid material and it influences the bone ingrowth because it allows migration and proliferation of osteoblasts and fosters vascularization.

The minimum pore size required to regenerate mineralized tissue is generally considered to be approximately 100 μm. However, pore sizes greater than 300 μm were observed to have a greater penetration of mineralized tissue in comparison with smaller pore sizes. At pore sizes of 75 μm, hardly any mineralized tissue is found within the scaffold. It is believed that for smaller pore sizes the penetration of neovascularization and nutrient supply to the growing cells is

slowed down. There are several criteria for the design of tissue engineering scaffolds that need to be taken into account: porous structure to support cell attachment, proliferation and extra cellular matrix production and, especially, interconnected pore network is very important in order to promote nutrient and waste exchange, also to control degradation rate of material [12]–[14]. Degradation rate is very important issue and in the best case degradation rate in terms of time has to be similar to the time of new bone formation. This means that in the space where previously biomaterial was implanted bone ingrowth takes place. This will lead to appropriate surface chemistry for cell attachment, proliferation and differentiation, mechanical properties to support equal those of the tissues at the site of implantation and a reproducible architecture of clinically relevant size and shape [15]–[17].

It is important that hydroxyapatite-endodontic cement scaffolds must be similar to the cancellous bone morphology and structure in order to provide integration in the surrounding tissue. Bone is a live structure composed of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ crystals deposited within organic collagen matrix.

Lots of scientists have tried to exploit hydroxyapatite in various combinations with other materials in order to get a substance which resembles hard tissue and can be proposed as bone substitute; it should match natural tissue design in order to enable close integration with the surrounding bone tissue in the body.

The purpose of this study is to create a biomaterial based on endodontic cement and natural hydroxyapatite, explore possibilities to use it as bone tissue substitute, investigate biological properties of this substance, affinity with live tissue and morphological alterations during implantation time.

II. Materials and Methods

Natural hydroxyapatite (NHAp) was obtained from bone tissue of cattle. Bone was cut into 2–4 mm thick layers, placed in furnace and heat-treated at temperature increasing from room temperature to 430 °C in 1.5 h. Next, specimens were kept at constant temperature for 5 h. After heat processing specimens were investigated for presence of protein. The presence of protein was ascertained by absorption method of infrared spectroscopy, wave interval 400–3500 cm^{-1}, using spectrometer *SCIMITAR 800 MIR* (USA); it showed that the protein was removed from the heat-treated specimens completely. Stripes of absorption which characterize protein – 1240 cm^{-1}, 1540 cm^{-1} and 1660 cm^{-1} were absent, but stripes of absorption which characterize the mineral part of bone tissue remained unchanged. After heat treatment the material was ground to fine powder. Specimens of the new biomaterial were prepared by mixing natural hydroxyapatite with *MTA Angelus* endodontic cement (EC). Two concentrations were used – first, NHAp 60 vol % and EC 40 vol %, second, NHAp 40 vol % and EC 60 vol %. Initially powders of both phases were mixed until homogeneous consistency, then water was added and mixing continued until homogeneous, gel like mass was obtained; the mixture have a consistency similar to wet sand. Next, this mass was filled into mold. The diameter of the mold was 5 mm, height 5 mm. Then specimens were left to harden for 72 h and afterwards taken out from the mold.

Samples were tested for mechanical characteristics. Modulus of elasticity, ultimate stress and ultimate strain in compression were determined and INSTRON-4301 testing machine (UK) was used to perform these tests. The experiments under uniaxial compression at strain rate of 0.5 $mm \cdot min^{-1}$ were carried out with *IMP 0.5* automatic testing machine controlled by *MTS* testing system (USA). The tests were continued up to the failure of specimens. Acquired experimental data of modulus of elasticity, ultimate stress and ultimate strain in compression were analyzed and compared with literature. Thirty specimens were tested and due to some imperfections in samples, ten best pieces from each group were selected to obtain an average value. Data of these tests are presented in the Table I.

In order to evaluate *in vivo* biocompatibility and process of resorption, materials were implanted subcutaneously in laboratory animals. For the experiment three months old male and female rats were used. The weight of laboratory animals on average was 200 ± 10 g. 0.008 mL of 0.1 % atropine, 0.3 mL of NaCl physiological solution, 0.075 mL of 0.05 % dexdomitor solution, 0.115 mL of 10 % ketamine solution was used as anesthetic. Two specimens were implanted subcutaneously in each rat. During the experiment laboratory animals showed neither any clinical signs of discomfort nor local inflammation reactions.

Twenty specimens were implanted, one sample of each concentration in one rat. During the experiment rats were given free access to food and water. Laboratory animals were sacrificed by overdosing ether narcosis after one, two and three month period of implantation. Specimens were harvested and their processing was done according to classical histological regulations, conventional hematoxylin-eosin (*Merck&Co*) staining was used. Investigation of the samples was done in reflected light with differential contrast microscopy using *Leica DMLP* microscope (Germany).

III. Results and Discussion

Obtained data gave valuable information for biomechanical, histological and biocompatibility analysis, although the overall knowledge is certainly not exhaustive, several facts have emerged clearly from these experiments.

On the basis of experimental results we can observe that material EC(60)-NHAp(40) which was implanted for one month in laboratory animals did not cause any inflammation reactions (Fig. 1).

Around the implanted specimen a connective tissue capsule had formed. It was possible to see that different cells, mainly connective tissue cells and connective tissue fibers had grouped around implant. Besides that, also small, newly formed blood vessels were presented; this indicates an adequate body reaction to implanted material. However, macrophages or any other cells usually involved in the process of phagocytosis and inflammation were not noted. Implanted material itself was still not resorbed and there were no signs of

decomposition, but only some small structural changes in it could be noted.

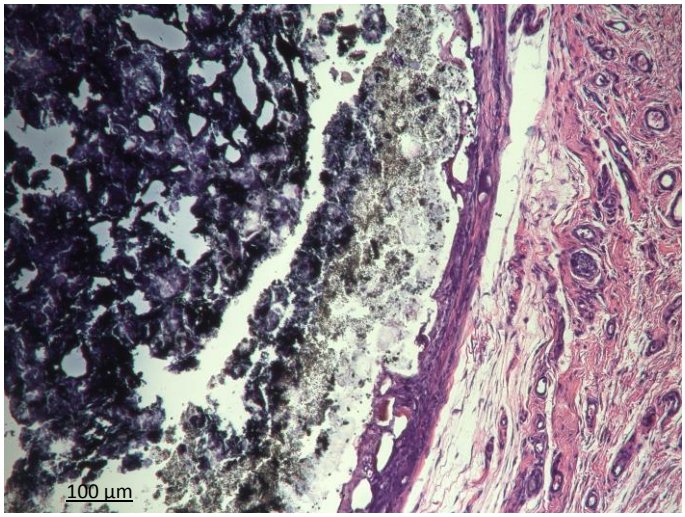

Fig. 1. Implanted material after 1 month, 100 × magnification.

Further, second material EC(40)-NHAp(60) with different proportions was also implanted for the same period of time (Fig. 2, Fig. 3). Histological picture and contact with surrounding tissue is similar to the first material. In this case material resorption also had not started and only slight changes could be noted.

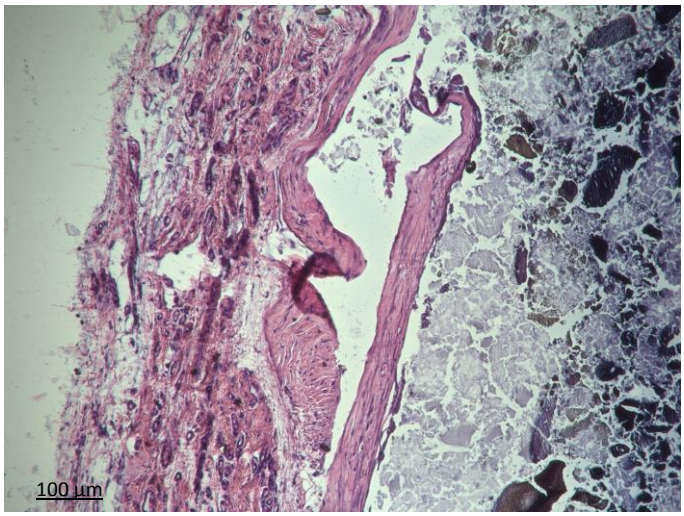

Fig. 2. Implanted material after 1 month, 100 × magnification.

Furthermore, if we observe material which has been implanted for two months, situation is different – a connective tissue capsule has formed around the implant and as well as before no inflammation reaction was noted. However, changes could be noted within the implanted material – it was possible to admit that structure of the material has started to degrade, Fig. 4.

In the picture above it is possible to see that the implanted material is not as dense as initially just after implantation. We can note structural changes in the material, this process shows that one fraction of material – natural hydroxyapatite – starts to resorb quicker; also literature data supports resorption peculiarities of different materials [18]–[20].

Next come implanted materials after implantation of 3 month period. In this specimen even more resorption of natural hydroxyapatite is expressed and material itself has become more porous (Fig. 5).

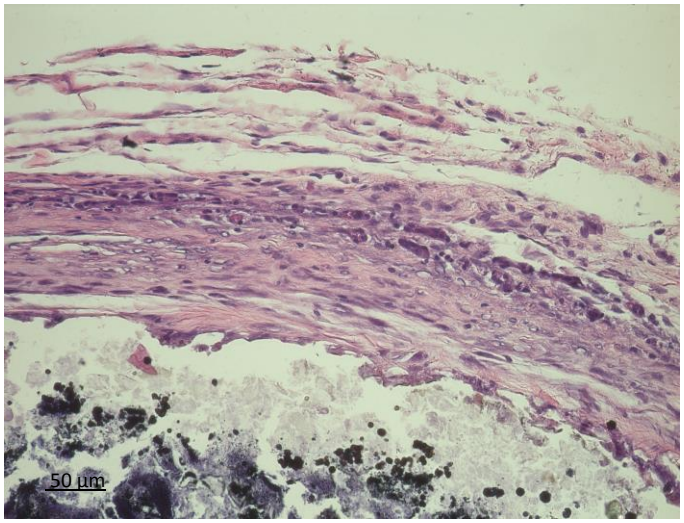

Fig. 3. Implanted material after one month, 200 × magnification.

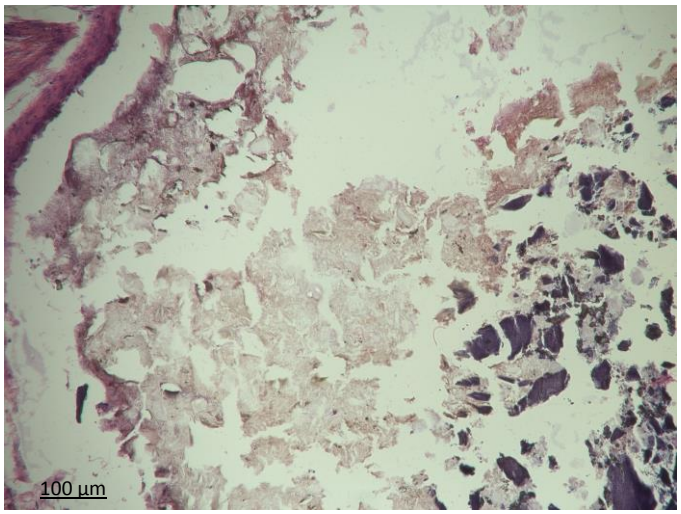

Fig. 4. Implanted material after two months, 100 × magnification. It is possible to note the formation of cavities between composite phases.

Furthermore described histological picture is very similar for both implanted materials, for the implantation period of two and three months. However, when the specimen was removed from the body of laboratory animal after experiment it had retained initial size and shape similar to specimen appearance before implantation; this consequently indicates that during three months of implantation material was not completely degraded, but it was possible to note that material has become more porous.

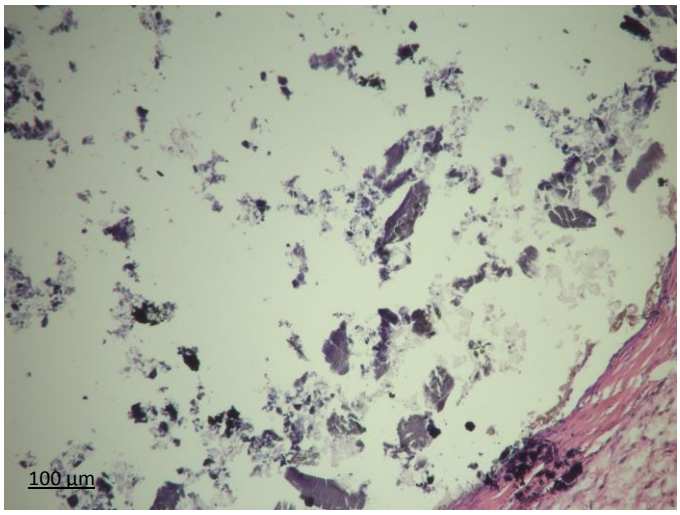

Fig. 5 Implanted material after three months, 100 × magnification. Formation of cavities continued and porosity of implanted material increased.

The mechanical properties of pure EC and EC-NHAp composite materials were determined as well. Specimens of these materials were loaded in compression at a strain rate of 0.5 mm·min^{-1}. The specimens were cylinders with 5 mm diameter of and 5 mm height. Based on experimental results the modulus of elasticity (E), ultimate stress (σ*) and ultimate strain (ε*) in compression were determined. These tests were continued up to the failure of the specimens. The results of the experiment are shown in Table I.

TABLE I

MODULUS OF ELASTICITY, ULTIMATE STRAIN AND ULTIMATE STRESS IN COMPRESSION

Material, (vol %)	**σ*, MPa, mean ± SD**	**E, GPa, mean ± SD**	**ε*, %**
EC(100)-NHAp(0)	47.42 ± 2.3	1.37 ± 0.19	0.035
EC(60)-NHAp(40)	16.69 ± 0.38	0.17 ± 0.03	0.1
EC(40)-NHAp(60)	9.75 ± 4.33	0.11 ± 0.04	0.11

Based on the obtained results we can conclude that greater percentage of filler – natural hydroxyapatite decreased modulus of elasticity and ultimate stress in compression, presumably, because mechanical properties of NHAp are weaker than properties of EC. Compared to the modulus of elasticity of compact bone this difference is even more expressed, these values for compact bone are within the range of 18–22 GPa in longitudinal direction and in transverse direction within a range of 5–13 GPa, ultimate elongation for compact bone is approximately 1.5 % and ultimate contraction – 1.8 %.

Based on positive biocompatibility reactions in the next step it is necessary to implant new biomaterial in compact bone in order to evaluate process what takes place on the bone/implant interface, strength of this bond and how it develops in longer time period. It is important also to follow up resorption process of implanted material and how it complies with the time when the new bone is growing into the implant. Next, in order to strengthen mechanical characteristics it is desirable to modify the content of the new biomaterial by adding reinforcing substance to improve parameters of ultimate strain and modulus of elasticity.

IV. CONCLUSION

Comparison between the composite materials made from EC-NHAp has shown that a change in the matrix filler ratio changes the mechanical characteristics and deformation properties of this material – greater percentage of natural hydroxyapatite filler decreases the modulus of elasticity and ultimate stress in compression.

Further, histological analysis of samples clearly indicates that during the implantation period of 3 months no inflammation reaction of host tissue was observed. Implanted material is covered by connective tissue capsule and cells responsible for inflammation reaction were not noted, neither macrophages nor any other cells usually presented in such situations. Consequently it is possible to assume that the new biomaterial is biocompatible with live bone and is accepted by host tissue.

Starting from the second month after implantation, it was possible to observe that resorption process of implanted material has been initiated. Initially, resorption of NHAp was more explicit and in the place where it was located previously formation of pores was noted. Size of new formed pores/cavities is within the range of 100– 200 µm. Consequently, in the case, if this biomaterial would be implanted into bone, it is expected that in these cavities bone inter-digitation can take place.

Results of our work have shown that EC-NAHp composites have sufficiently good mechanical properties and biocompatibility in order to be used as implant materials for covering bone defects and for creating new bone structure after surgical interventions. Advantage of the new material is quick setting time due to the presence of endodontic cement in it. This substance also provided good fixation of implanted material and later gradual resorption time creating space for ingrowth of natural hard tissue.

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Lauris Rupeks, Viktors Fiļipenkovs, Ivars Knēts, Visvaldis Vītiņš, Marina Sokolova, Līga Stīpniece, Māra Pilmane. Uz deproteinizēta hidroksiapatīta un endodentīna cementa bāzes veidota biomateriāla biosaderība un mehāniskās īpašības.

Hidroksiapatīts tiek lietots kaulaudu rekonstrukcijai, tā mehānisko īpašību uzlabošanai pievieno citas vielas. Šajā darbā biomateriāls ir veidots uz dabiska deproteinizēta hidroksiaptīta un endodentīna cementa bāzes, un noteiktas tā mehāniskās īpašības. Materiāls tika implantēts laboratorijas žurku zemādā un veikta histoloģiskā analīze kā arī noteikta biosaderība. Rezultāti rāda, ka materiālam ir piemērotas mehāniskās īpašības kaula sanācijai, īss cietēšanas laiks un pakāpeniskas rezorbcijas rezultātā veidojas poras, kas ir piemērotas kaulaudu integrācijai.