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Rīgas Stradiņa universitāte

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CHARACTERISTICS OF REACTOGENICITY IN DISTANT TIME PERIODS AFTER IMPLANTATION OF BONE TISSUE SUBSTITUTING BIOMATERIALS

Summary of Doctoral Thesis
for obtaining the degree of a Doctor of Medicine
Speciality – Morphology

Riga, 2016

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The Doctoral Thesis was carried out at the Institute of Anatomy and Anthropology, Rīga Stradiņš University

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TABLE OF CONTENTS

ABBREVIATIONS USED

Abbreviation	English	Latvian
IL-1	interleukin-1	interleikīns-1
IL-6	interleukin-6	interleikīns-6
IL-8	interleukin-8	interleikīns-8
IL-10	interleukin-10	interleikīns-10
TNF-α	tumor necrotic factor-α	audzēja nekrotiskais faktors-α
βDef-2	β-defensin-2	β-defensīns-2
βDef-3	β-defensin-3	β-defensīns-3
βDef-4	β-defensin-4	β-defensīns-4
BMP-2/4	bone morphogenetic protein-2/4	kaula morfoģenētiskais proteīns-2/4
OPG	osteoprotegerin	osteoprotegerīns
OP	osteopontin	osteopontīns
OC	osteocalcin	osteokalcīns
TUNEL	terminal deoxynucleotidyltransferase dUTP nick end labelling	gala deoksinukleotidiltransferāzes un digoksigēna marķēti nukleotīdi
HAP-1	hydroxyapatite-1, sintered at 1000 °C	hidroksiapatīts, apdedzināts 1000 °C
HAP-2	hydroxyapatite-2, sintered at 1150 °C	hidroksiapatīts, apdedzināts 1150 °C
β-TCP-1	β-tricalcium phosphate-1, sintered at 1000 °C	β-trikalcija fosfāts-1, apdedzināts 1000 °C
β-TCP-2	β-tricalcium phosphate-2, sintered at 1150 °C	β-trikalcija fosfāts-2, apdedzināts 1150 °C
α-TCP cements I	α-tricalcium phosphate cement I with pH 6.0	α-trikalcija fosfāta cements I ar šķidro fāzi pH 6,0
α-TCP cements II	α-tricalcium phosphate cement II with pH 7.0	α-trikalcija fosfāta cements II ar šķidro fāzi pH 7,0
α-TCP-pH 6.0	α-tricalcium phosphate cement with pH 6.0	α-trikalcija fosfāta cements ar šķidro fāzi pH 6,0
α-TCP-pH 7.0	α-tricalcium phosphate cement with pH 7.0	α-trikalcija fosfāta cements ar šķidro fāzi pH 7,0

α-TCP-pH 8.0	α-tricalcium phosphate cement with pH 8.0	α-trikalcija fosfāta cements ar šķidro fāzi pH 8,0
HAP/β-TCP-1	biphasic hydroxyapatite/ β-tricalcium phosphate-1, sintered at 1000 °C	bifāzisks hidroksiapatīts/ β-trikalcija fosfāts-1, apdedzināts 1000 °C
HAP/β-TCP-2	biphasic hydroxyapatite/ β-tricalcium phosphate-2, sintered at 1150 °C	bifāzisks hidroksiapatīts/ β-trikalcija fosfāts-2, apdedzināts 1150 °C
HAP/PCL	hydroxyapatite covered by polycaprolactone	ar polikaprolaktonu pārklāts hidroksiapatīts
PMMA	polymethylmethacrylate	polimetilmetakrilāts
Bcl-2 gēns	B cell lymphoma-2 gene	B šūnu limfomas-2 gēns
RTU Rūdolfa Cimdiņa RBIAC	Riga Tehnical university Rudolfs Cimdins Riga Biomaterials Innovation and Development Centre	Rīgas Tehniskās universitātes Rūdolfa Cimdiņa Rīgas biomateriālu inovāciju un attīstības centrs

1. INTRODUCTION

Topicality of study

The use of surgical implants in stomatology and orthopaedics was introduced in the 50ties of the last century and, in parallel to it, research was going on, mainly for the improvement of their qualitative properties (*Ratner*, 2013; *Schminke et al.*, 2015). Despite it, the research for the possibility to use the bone tissue substituting biomaterials in stomatology and orthopaedics is still continued, and it is an important issue in the world and in Latvia as well.

Bone cements for the correction of different kind of bone defects are used as substitution materials of bone transplants for strengthening of joint replacements, for the growth of bone mass, as well as for the supply system of local medicines, and manufacturing of cell matrix in bone tissue engineering (*Shi*, 2006; *Talmadge*, 2006; *Habraken et al*., 2007; *Kokubo*, 2008; *Ratner et al*., 2013). In stomatology practice different type materials are used for biomaterial research. Mostly they are calcium phosphate-containing materials – hydroxyapatite (HAP), which can be either pure, or coated, polyesters polycaprolactone (PCL) or polylactate acid (PLA). Like a bone cement component, there is used also methylmethacrylate and/or polymethyl-methacrylate (PMMA) powder, or HAP-chitosan-PMMA combinations and other materials.

In the Republic of Latvia there are also carried out chemical studies on bone tissue substituting materials. Thus, in Riga Technical University Rudolfs Cimdins Riga Biomaterials Innovation and Development Centre (RBIDC) the work is going on the development of hydroxyapatite, biphasic calcium phosphate, polymethylmethacrylate (PMMA) cement, glass ionomer cement and calcium phosphate bone cement synthesising and their characteristic property research. Out of them, PMMA and ceramic materials of different

calcium phosphate combinations were used also in the current study author's research with experimental animals. Any substance implantation in biological tissues proceeds with mechanical damage and their consequent response reaction, tissue healing and regeneration process whose length is affected by individual mechanisms of immune defense system, and which can vary also due to affected tissue locality (*Konig et al.*, 1999; *Leite and Ramalho*, 2008). Reaction of immune system cells, mainly reaction of macrophages on biomaterial, is attached prognostic importance in the formation of granulation tissues and foreign body type reaction process, and also in biomaterial functionality (*Brown et al.*, 2012; *Laurencin and Khan*, 2013). It is essential therefore to determine also cytokine interleukin-10 (IL-10) and also IL-1, IL-6 and IL-8 expression in relation to biomaterial implantation. As a result of the activity of inflammatory mediators – both proinflammatory IL-1, IL-6 and IL-8, and antiinflammatory IL-10, the inflammation usually occurs at the initial period and causes cell apoptosis – cell death as well.

An essential role in the tissue regeneration process is played also by the chosen implant material, which, as described by *Yamaguchi et al.* (1997) and *Anderson* (2001), in its turn, is one of its biocompatibility formation components. They are also explaining that in biocompatibility formation process an essential role is attributed to the mutual interaction of immune system reactions with the biomaterial and post-implantation period. It is still important to create biocompatible materials, which, logically, call for the necessity to do parallel studies on tissue reactogenicity. *Slutski and Vetra* (1996) have worked out the reactogenicity theory, dividing tissue reaction of implantation into nonspecific and specific, which together make the biocompatibility. Tissue reactogenicity is the body local reaction which involves in itself adaptive mechanisms, and the immune system reactions are considered a component of this process (*Szebeni*, 2012).

Tissue reactogenicity markers, i.e., tissue reactions and released substances in the implanted region, the growth factors as well, cell death parameters and inflammatory mediators have an essential role in this response reaction of the body. Tissue nonspecific and specific reactogenicity is identified also at immunohistochemical examination level. Thus, tissue nonspecific reactogenicity markers are cytokins IL-1; IL-6; IL-8; IL-10, antimicrobial activity markers – β-Defensin-2 (βDef-2); βDef-3; βDef-4, but bone tissue specific reactogenicity markers are bone morphogenetic protein (BMP), osteoprotegerin (OPG), osteopontin (OP) and osteocalcin (OC) (*Lee*, 2000; *Sarfati*, 2004; *El-Ghannam*, 2005). In the literature, however, there are little data described on studies of tissue nonspecific and specific reactogenicity markers expression in the implant's zone and their role in determination of biocompatibility of implantation material in distant time periods. It is essential to state, when nonspecific reactogenicity ends and, what the dynamics of bioactivity or specific reactogenicity is. The role of βDef-1, βDef-2, βDef-3 in protection of immune system reactions against infections in cases of biomaterial implantations is not completely defined, and one cannot find exact data in the literature on cytokins and defensins expression in bone tissues around different type of bioceramic implants. Therefore, morphological changes in the implantation region are still unclear, and no prognostic criteria of morphologicall diagnostic are defined for the determination of the tissue degeneration and regeneration in implantology.

Aim of study

The aim of the study was to analyze morphological factors of local reactions in experimental animals' tissues at different periods of time after the implantation of bone tissue substituting biomaterials.

Objectives of study

1. To analyze study samples for tissue biocompatibility in hard and soft tissues around different type biomaterials in experimental animals' tissues and in a control case.

2. To analyze the expression of proinflammatory and antiinflammatory markers' cytokine - IL-1; IL-6; IL-8 and IL-10 in hard tissues at distant time periods (3, 4.5, 6 and 8 months) after implantation of different biomaterials.

3. To analyze the expression of antimicrobial protein βDef-2 in hard tissues at distant time periods after implantation of different biomaterials.

4. To analyze the bone morphogenetic protein BMP-2/4 and the expression of cell functional activity marker OPG in hard tissues at distant time periods after implantation of different biomaterials.

5. To analyze the expression of protein of the bone matrix proteins – OP and OC in hard tissues at distant time periods after transplantation of different biomaterials.

6. To determine the distribution of proinflammmatory, antiinflammatory, antimicrobial and bone growth factor, cell functionality marker, and bone matrix proteins in the hard tissues of experimental animals.

7. To determine the distribution of apoptosis markers in the hard tissues of experimental animals at distant time periods after implantation of different biomaterials.

8. To analyze correlation of compatibility markers at different periods of time after implantation and with the control data.

9. To develop morphologically prognostic reaction algorithm for stating tissue biocompatibility at specific (3, 4.5, 6 and 8 months) distant time periods.

Hypotheses of study

1. Tissue local reactogenicity is different at different periods of time after implantation of bioceramic material substituting bone tissue calcium phosphate.

2. Calcium phosphate bioceramic implants of different content and structure have a selective inductive effect in tissue molecular processes.

Novelty of study

At a more distant time period (after 3, 4.5, 6 and 8 months) after implantation of different calcium phosphate materials and polymers, there have been determined the differences of expressions of biomarkers of tissue nonspecific and specific reactogenicity for the development of morphologically-diagnostic algorithm. For the first time the expression differences of the factors were described, the explanation was given and the markers defined to be used at each implantation period.

Individual contribution

The author of the current study has carried out routine morphological examination of tissue samples, the immunohistochemical assessment and visualization, as well as she is the author of all microphotographs.

Ethical aspect

For the undertaking of the current study there has been issued the permission by the Latvian Food and Veterinary Service (Nr. 24, 02.07.2010.).

Structure and volume of study

The doctoral thesis is written in the Latvian language. It has a classical structure and it contains 8 parts: introduction, the literature review, material and methods, results, discussion, conclusions, the list of literature used and appendices. The doctoral thesis comprises 152 pages, including 18 tables, 7 pictures-diagrams, 66 microphotographs. 214 reference sources have been used (in summary – 207 sources).

2. MATERIAL AND METHODS

2.1. Study material

For the study of tissue reactogenicity for the biomaterial implantation were used experimental animals – rabbits of California. 19 animals were included in the study. One side mandibular or shin bone tissues of the animals were chosen for biomaterial implantation, but the other side mandibular or shin bone tissues and soft tissues for the formation of control samples.

Biomaterial implantation operations were done in 4 stages, which were respectively divided into four experiments. Using the general anaesthesia and local anaesthesia there were done biomaterial implantation operations (Ģ. Šalms, A. Skaģers). According to the planned implantation terms (in the 1st experimental group – in 6 and 8 months, in the 2nd and 3rd experimental group – in 3 months, and in the 4th experimental group in – 4.5 months), using the air embolization method the animals underwent euthanasia, the tissue block from biomaterial implantation zone and from the control side were identified. In total 33 experimental samples were obtained for morphologic examination from biomaterial implantation zone and 12 control samples.

For the study there were used noncommercial – RTU Rudolfs Cimdins RBIAC developed biomaterial implants (prepared by J. Ločs and V. Zālīte) and commercial:

1. In the 1st experimental group – sintered at 1150 °C hydroxyapatite granules (**HAP-2)**;
2. In the 2nd experimental group – **commercial PMMA** bone cement (*Biomet Bone Cement R; Biomet Inc., Switzerland*), **noncommercial PMMA** cement, **α-TCP cement I** (with the initial liquid phase pH 6.0), **α-TCP cement II** (with the initial liquid phase pH 7.0), unsintered **HAP granules,** sintered at 1150 °C **HAP**

granules (HAP-2), **with polycaprolactone** 30% (PCL) **coated HAP tablet**, **uncoated HAP tablet**;

3. In the 3rd experimental group – sintered at 1000 °C hydroxyapatite/β-tricalcium phosphate granules **(HAP/β-TCP-1)**, sintered at 1150 °C hydroxyapatite/β-tricalcium phosphate granules **(HAP/β-TCP-2)**, sintered at 1000 °C β-tricalcium phosphate granules **(β-TCP-1)**, sintered at 1150 °C β-tricalcium phosphate granules **(β-TCP-2)**, sintered at 1000 °C hydroxyapatite granules **(HAP-1)**;
4. In the 4th experimental group – **α-TCP cement pH 6.0, α-TCP cement pH 7.0, α-TCP cement pH 8.0**.

2.2. Study methods

2.2.1. Morphological study methods

In the study there were used the classical tissue preparation and morphofunctional examination methods, carried out at Rīga Stradiņš University Institute of Anatomy and Anthropology, within the framework of the national research "Development of Innovative, Multifunctional Materials, Signal Processing and Informatics Technologies for Competitive Scientific Products), project 4 "Innovative materials and technologies for biological tissue assessment and substitution" (M. Pilmane, N. Moroza).

After release of the bone tissue block, tissues were fixed in *Sol. Stefanini* not less than 24 hours. After fixation the bone tissues were decalcinated by *Decalcifer rapid* solution. Then the tissues were dehydrated, using ethyl alcohol from 70° till 96°, and defatted in xylene solution. Then there followed the placing of tissues into cassettes with paraffin, using the so-called paraffin blocks, of which were made 3–5 μm thick sections and were

applied on slides for their further processing and staining. To acquire the **review of the morphologic picture** of the morphologic investigation of bone tissues and soft tissues, we used routine examination method with hematoxylin/eosin (*Kiernan et al.,* 2008). Tissue immunohistochemical staining for **biomarkers identification** was done by biotin-streptavidin method (*Hsu et al*., 1981), using the following **βDef-2, IL-1**, **IL-6, IL-8, IL-10, BMP-2/4, OPG, OP, OC** antibodies. Cell **apoptosis** was determined using Caspase-6 antibody by biotin-streptavidin method (*Hsu et al*., 1981) and by TUNEL method (*Gavrieli et al.,* 1992). For the assessment of relative frequency of parameters/markers in bone tissues, determined immunohistochemically and by TUNEL method there was applied a widely used, and widely described method in the literature – semi-quantitative counting method (*Tobin et al*., 1990; *Pilmane et al*., 1998). Factor expression was analyzed in three randomly selected visual fields of one section (Table 2.1).

Table 2.1

Marking of relative frequency of positive structures identified immunohistochemically and by TUNEL method

–	Positive structures are not found in visual field
+	Few positive structures in visual field
++	Moderate amount of positive structures in visual field
+++	Numerous positive structures in visual field
++++	Abundance of positive structures in visual field

For **visualization** of morphological findings there was used light microscopy and photography by microscope *Leica DMRB* and the processing by computer programme *Image-Pro Plus*.

2.2.2. Statistical processing methods

In the data statistical analysis there were used descriptive and analytical statistical methods. For the assessment of the differences as to the occurrence of the markers of morphological findings there were used nonparametric data analysis tests, i.e., *Mann–Withney* and *Wilcoxon* tests. For the description of immune-positive cells and apoptosis there were used the mean arithmetic and dispersion indices (standard deviation and standard error). In the study there was used the significance level α=0.05. Thus, if in the statistic test calculations p value was less than 0.05 ($p<0.05$), the results then were considered to be statistically significant. To evaluate correlation closeness of two variables, there was used *Spearman's* correlation method. Correlation coefficient $r_{s,}$ like a correlation closeness quantitative index between two or more variables, was calculated as *Spearmen's* correlation coefficient: correlation coefficient from 0.7 till 0.9 – close correlation value; correlation coefficient from 0.3 till 0.7 – moderate correlation; lesser than 0.3 (0.1–0.3) –weak correlation.

By one or two asterisks*, ** there was marked significant correlation. Statistical analysis was done by means of computer programme IBM SPSS 20 (SPSS Inc., USA).

3. RESULTS

3.1. Morphological findings and data statistical analysis in the 1st experimental group

In soft tissue review sections, either in 6, or 8 months' implant HAP-2 region there were found similar changes in the tissue structure – dystrophy and new formations of separate skeletal striated muscle fibres, here and there with inflammatory cells, mainly, lymphocyte infiltration endo- and perimysium. In soft tissues of the control zone the mentioned changes were little expressed. In one of the bone tissue samples, after 6 months, around HAP-2 there was found the granulation tissue containing capsule. In bone tissue review sections in the control zone after 6 and 8 months, there was found a partial or complete obliteration of osteon canals with connective tissues and separate blood-vessel sclerosis. Immunohistochemical examination found that βDef-2, BMP-2/4, OPG, OP, OC, cytokines – IL-1, IL-6, IL-8 and IL-10 containing cell number in bone tissues 6 and 8 months after HAP-2 implantation is different both in the experiment, and control tissues. The most interesting data were found 8 months after implantation. For instance, the greatest average number of cells was found in one of the bone tissue samples with OPG marker expression (37.33±0.88) 8 months after HAP-2 implantation, as well as in one of the control tissue samples (37.00±0.57). Eight months after implantation a comparatively great number of BMP-2/4 expressing cells (23.66±0.33) was found also in a control sample, which was greater than in a sample with a biomaterial (13.00±0.57). The average number of interleukin expressing cells was variable. IL-6 expressing cells of the experiment sample 8 months after implantation made the greatest number (24.33±2.40), which was great also in a control group (22.00±1.15). IL-10 expressing cells made a similar number both in the experiment (23.66±2.02), and in the control group (23.33±1.85) 8 months after

implantation. A greater average of apoptotic cell count both in the experiment, and in the control group was seen in 6 months. In the data statistical analysis βDef-2, IL-1, IL-6, IL-8, IL-10, BMP-2/4, OPG, OC and OP containing cells, as well as average number of apoptotic cells did not statistically significantly differ in the experimental and control group, $p>0.05$.

3.2. Morphological findings and data statistical analysis in the 2nd experimental group

After three months, soft tissues in the implant zones were found to have tissue edema, separate lymphocyte infiltration and fibroblast proliferation, which was lesser expressed in α-TCP cement and noncommercial PMMA cement area. The formation of a new bone with the bone-implant incomplete coalescence signs was found in all implant areas, except for a noncommercial PMMA cement area. The average number of antimicrobial protein βDef-2, interleukins IL-6, IL-8 and IL-10, and also BMP-2/4, OPG, OC and OP expressing cells in the experiment and control tissues was variable. The greatest average number of βDef-2 expressing cells (32.66±1.45), IL-6 (36.33±1.45), OPG (31.33±0.33) and OP (34.33±2.02) was found in samples with PCL coated HAP implantation zone. At the same time, in the bone tissue samples from α-TCP cement I implantation zone, there was found the greatest average number of IL-1 positive osteocytes (31.66±1.20), but the OC expressing cells (37.00±2.08) – in the area of noncommercial PMMA. In the samples with commercial PMMA cement, there was found the greatest number of IL-8 expressing cells (24.00±1.73) and the number of BMP-2/4 expressing cells (48.00±1.15). The greatest average number of proinflammatory cytokine IL-10 expressing cells (35.66±1.76) was found in the samples with uncoated HAP. In the control group the greatest number of expressing cells was made by IL-6 positive cells (27.33±1.95), and the least number – also IL-8 expressing cells.

The number of IL-1 containing cells in the experiment group *(Z=1.77; p=0.04)* was found to be more statistically significant. The average number of apoptotic cells in bone tissues was from 1.3±0.57 apoptotic cells in α-TCP cement II area till 48.3±5.50 apoptotic cells in the area of unsintered HAP granules. Apoptosis in bone tissues in the control area, in its turn, was not observed.

3.3. Morphological findings and data statistical analysis in the 3rd experimental group

In three months, in the experiment samples with β-TCP-2 and HAP-1 granules, we found soft tissue changes with an expressed amount of macrophages and fibroblasts. In bone tissues, three months after HAP/β-TCP-1, HAP/β-TCP-2, β-TCP-2 and HAP-1 granules implantation, there were found bone tissue resorption zones and the formation of a new bone. The majority of IL-1 positive osteocytes are found in bone tissue samples from HAP-1 implantation zone (21.33±0.88). The greatest average number of IL-6 positive cells is seen in β-TCP-1 biomaterial area (15.33±1,20). IL-8 positive cells are found only in β-TCP-2 area, and their number was comparatively small (2±0) as well. The average number of IL-10 positive cells, in comparison to other cytokines, was comparatively low, but the greatest of them (12.00±0.57) was seen in HAP-1 area. βDef-2 was found only in HAP-1 area and the average number of positive cells of this biomarker in three visual fields was 12.00±0.57, which was close to that of the control (2.33±0.33). The average greatest number of OP and OC expressing osteocytes (OP – 31.66±0.88; OC – 34.00±0.57) was found in HAP-1 area. The greatest average number of BMP-2/4 positive osteocytes was found in β-TCP-2 area (22.66±0.88), which was more than the average number in the control tissues (1.67±1.67). OPG expression was comparatively less marked in the samples with β-TCP-1 and HAP/β-TCP-1. In

the control group, in its turn, the greatest number was made by OP positive osteocytes (18.67±3.67). **The number of *BMP-2/4* containing cells in the experiment group *(Z=1.84; p=0.03)* was found to be more statistically significant**. The greatest average number of apoptotic cells in three visual fields was seen in HAP-1 implantation area (65.00±21.18), but in the comparison of the experiment and control group there do not exist a statistically significant difference as to the number of apoptotic cells.

3.4. Morphological findings and data statistical analysis in the 4th experimental group

4.5 months after α-TCP (with pH 6.0, pH 7.0 and pH 8.0) implantation there were found in the biomaterial areas the granulation tissues and fibroses zones, and zones of the formation of a new bone. In the control tissues there were also found small granulation tissue areas. Biomarker expression after 4.5 months was comparatively little marked. In bone tissues with tricalcium phosphate material, the greatest biomarkers-expressing cells were made by BMP-2/4 positive cells (1.19±0.44), but the least number was made by IL-1 positive osteocytes (0.11±0.11). In the control group the average number of OPG expressing cells was 2±0, which were also the only immune positive cells in this group. In the 4th experiment the number of biomarkers-containing cells in the experiment and control group did not statistically significantly differ, $p>0.05$. The number of apoptotic cells in bone tissue samples after α-TCP implantation was variable and it was found most of all in the sample with α-TCP-pH 6.0 (44.33±2.33). In the number of apoptotic cells no statistically significant differences were found.

3.5. Intercorrelation of biomarkers

Analyzing Spearman's range correlation coefficients between immuno-histochemical markers in **control bone tissues,** we found the following a **statistically significant close correlations** after ***3 months*** only:

1. With the increase of ***IL-1*** release in osteocytes, there increased also ***OP*** release, (r_s=0.880);

2. With the increase of ***IL-6*** release in osteocytes, there increased also ***OPG*** release, (r_s=0.897);

3. With the increase of the number of ***IL-8*** containing osteocytes, there increase also the number of ***βDef-2*** containing osteocytes, (r_s=0.897).

Analyzing Spearman's range correlation coefficients between the factors in **the experiment bone tissues**, a **statistically significant close correlations** we found ***3*** **and** ***4.5 months*** **after biomaterial implantation**.

3 months after implantation:

1. With the increase of ***βDef-2*** expression in osteocytes**,** there increased also ***IL-10*** release (r_s=0.703), ***OP*** (r_s=0.755, r_s=0.705) and ***OC*** release (r_s=0.701);

2. With the increase of ***IL-1*** release in osteocytes, there increased also ***IL-10*** release (r_s=0.810);

3. With the increase of the number of ***IL-6*** expressing osteocytes, there increased the number of ***OP*** expressing osteocytes, r_s=0.738);

4. With the increase of the number of ***IL-10*** containing osteocytes, there increased the number of ***OP*** containing osteocytes (r_s=0.780);

5. With the increase of the number of ***OP*** expressing osteocytes, there increased also the number of ***OC*** expressing osteocytes, (r_s=0.944).

4.5 months after implantation:

1. With the increase of the number of **βDef-2** expressing osteocytes, there increased ***OC*** expressing osteocytes, (r_s=0.711); and with the increase of the number of **IL-1** expressing osteocytes, there increased also the number of ***OC*** expressing osteocytes.

4. DISCUSSION

4.1. Morphological findings of routine examination at different periods of time after biomaterial implantation

In our research, ***3 months*** after the implantation of biomaterials there were found nonspecific tissue reaction manifestations in the soft tissues, such as tissue edema and single lymphocyte infiltrations that could be related to the inflammatory process. Marked morphological changes mentioned were observed either in "pure" HAP, or with PCL coated HAP tablets, or PMM bone cement areas. In soft tissues, in their turn, after ***4.5 months*** active inflammation, the process had decreased, but there were seen its consequences in the form of granulation tissues and fibrosis. The most expressed granulation tissues and fibrosis around the implantation area had resulted from the implantation with α-TCP (with pH 6.0, pH 7.0 and pH 8.0), stating that there had likely been a catarrhal inflammation around these implants, too. Besides, ***6*** and ***8 months*** after sintered HAP-2 implantation there had been observed dystrophy of separate muscle fibres with single lymphocyte infiltration areas, which, in their turn, indicated to the degenerative tissue changes. The mentioned morphological changes, though less expressed, were observed also in the soft tissues of the control area, however, only in some visual fields. In our study the morphological findings in implantation periods prove about the nonspecific tissue response reaction on biomaterial implantation, which, in some cases (for instance, sintered HAP-2) transfer even into degenerative changes. Our findings coincide with the data of other authors on the fact, that wound healing and tissue reactions due to foreign bodies are considered to be the general tissue reactions to the damage. *Shaposhnikov* (1997) and *Robbins et al.* (2005) have mentioned, that, starting already from 2–3 weeks, there are being observed such soft tissue healing signs as scarring and formation of

granulation tissues that can proceed with the development of new blood-vessels and stroma fibrosis. It is interesting here to point also to the observations of other authors as well. For instance, *Miyatake et al.* (1989) had observed, that in patients after HAP implantation the wounds healed and the inflammation disappeared within a month after the operation. *Jones et al.* (2001) had observed, that 8 weeks after implantation of uncoated polyethylene and with PMMA particles coated polyethylene into the dog's shin, there was developing a fibrous capsule around implants, which increased with the increase of the postimplantation time, i.e., after 12 and 24 weeks. He found also cytokines IL-1 and IL-6 expression in tissues, which was related to the immune system response, reaction to osteolysis.

Bagambisa et al. (1994) and *Anderson* (2008) have found, that the development of the inflammation in implant's areas are related to the development of acute or chronic inflammation in these areas, which is mostly depending on the implant's physically chemical properties. But, *Mosser* and *Edwards* (2008), *Brown et al.* (2012) in this context point also to proinflammatory cytokine activities, the link of the process and intensity of wound healing and fibrosis with the local condition of certain tissues and the implant's chemical signals, which, to our mind, was the decisive factor in our study. The authors also say, that, the more biocompatible is the implant, the less expressed are the body response reactions.

All in all, morphological findings in our study are connected with an individual soft tissue response reaction to trauma, and partially also to selective soft tissue reaction to a certain biomaterial (sintered HAP-2). The intensity of the manifestation of response reaction depends also on the chosen biomaterial adequacy and/or tissue damage depth during the implantation procedure. In our study the degenerative bone tissue changes, i.e., a new bone formation, was observed already ***3 months*** after implantation. In bone tissues, within 3 months, just after implantation of HAP tablet, with PCL coated HAP tablet, PMMA

cement and unsintered HAP granule implantation, there were found extensive new bone formation zones. In the areas of other biomaterials in this postimplantation period, the areas of the new bone were not expressed, but after ***4.5 months*** we found them also in α-TCP with pH 6.0, pH 7.0 and pH 8.0 implantation case. But ***6 and 8 months*** after implantation we did not find a new bone formation signs in HAP area. The findings in our study proved the prevalence of the bone tissue regeneration for ***3 months*** after implantation, which coincides with other authors' data given on postimplantation tissue regeneration. However, in different biomaterial implantation cases, the onset of bone tissue regeneration is observed at different time intervals, and, sometimes, there can be found also contradictory data on the osteogenesis onset in the areas of certain biomaterials. For instance, *Harada et al.* (1989), implanting HAP/TCP in the rabbit's mandibular bone, observed the new bone formation signs on the implant's surface already one week after implantation, but after 48 weeks – a new bone growth between HAP pores. *Shaposhnikov* (1997), *Robbins et al.* (2005), *Chen et al.* (2015) have noticed, that in the cases of damage of different type supportive tissues the bone tissue scarring and a new bone formation occurs strating from 2-3 weeks. It is interesting to mention also *Chazono et al.* (2008) study, where he had found 2–3 weeks after β-TCP implantation into the femur of rabbits of New Zealand the accumulation of lymphocyte, monocyte and giant cells on the surface of the newly formed bone next to the biomaterial, but after 4 weeks – a new bone formation with β-TCP micropores in it. But (2010) and *Lee et al.* (2011) have described, that the new bone formation after HAP implantation had been found both 3 months, and 6-12 months after the biomaterial implantation. *Baslé et al.* (1993) and *Roldán et al.* (2010), using calcium phosphate implants, found the bone formation signs not only 3 months after implantation (like in our case after HAP, with PCL coated HAP tablet, PMM cement and unsintered HAP granule implantation), but in a much earlier period too, i.e., one week after implantation.

An interesting seems to be *Moghadam et al.* (2004) observation on a new bone formation, which has been noticed by the authors both 6, and 12 weeks after calcium phosphate cement material implantation into the New Zealand rabbits' skull, but a more expressed, though without a significant difference within the implanted and control areas, it was seen in 12 weeks or 3 months' implantation period. In the study with biphasic calcium phosphate *Froum et al.* (2008), 6–8 months after biomaterial implantation, the patient's trepanation biopsy material's histomorphometric examination revealed a new bone formation, in such a way identifying the osteoconductive properties of this biomaterial. In our study we have also observed a bone formation in the area of biphasic calcium phosphate material - HAP/β-TCP-1 and HAP/β-TCP-2, but less expressed after ***3 months***. *Tsai et al.* (2010), in the period of 6–12 months after HAP implantation in patients with comminuted bone fractures, benign bone tumours with cavity formation had observed, that in 81,8% cases there was found bone tissue mass formation radiographically, and microscopically – a new bone formation on the implanted HAP material.

In general, considering these facts, the bone tissue formation proceeds mainly in the period of ***3 and 4.5 months*** after the biomaterial implantation, pointing to the start of bone tissue regeneration prevailing for ***3 months*** after implantation.

4.2. Data of immunohistochemical findings at different post-implantation periods after different biomaterial implantation

4.2.1. Morphogenetic bone protein-2/4 (BMP-2/4)

In our experimental animals in four experiments after different biomaterial implantations BMP-2/4 expressing cells were the ones of the most frequently encountered cells, in comparison to other biomarkers expressing cell

count. However, the number of BMP-2/4 expressing osteocytes in the animal bone tissues was variable, with the greatest prevalence for ***3 months*** after implantation with PCL coated HAP tablet's area, and then in β-TCP and unsintered HAP granule area. BMP-2/4 containing osteocyte count in the experiment and control group in ***3 months*** also statistically significantly differed, what was not observed in other implantation periods. The greatest number of BMP-2/4 expressing cells, which was observed comparatively early after implantation, i.e., ***3 months***, it is similar also to other authors' observations on BMP activity and shows BMP stimulating role in the bone tissue healing process. In the studies of *Aebli et al.* (2005), *Saito et al.* (2005) on BMP stimulating effect on bone tissue regeneration and *Fu et al.* (2008) in the study on bone tissue segmental defect healing have noticed, that HAP particles regulate BMP-2 release and HAP high concentration even stimulate it. Also *Yu et al.* (2010) have pointed, that one of the BMP essential roles is the participation in osteoblast differentiation and bone formation process and it is essential just in the early stages of the fracture healing, when BMP activates bone progenitor cells in periosteum and endosteum. The mentioned *Yu et al.* (2010) observations correspond also to our findings ***3 months*** after implantation, which, on average, corresponds to the early phase of the fracture healing. ***4.5 months*** after implantation the number of BMP-2/4 positive osteocytes was already lesser, however, with a different pH α-TCP in all samples. We should say, that ***4.5 months*** after implantation we observed a close statistically significant correlation between BMP-2/4 and βDef-2, OC, IL-1 and IL-10 release, although also at this time the release of these factors was comparatively little expressed, and it characterizes the exhaustion of functions. In our study, ***6 months*** after sintered HAP-2 implantation, there was observed a slight prevalence of the growth factors' expressing osteocytes, i.e., moderately enough, in the biomaterial implantation area, but after ***8 months*** they mostly were located in the tissue control area. We did not observe any

statistically significant differences in the number of this factor containing cells, nor a statistically significant correlation with other factors within ***6 and 8 months***.

To our mind, a comparatively lesser number of BMP-2/4 positive osteocytes during ***4.5, 6*** and ***8 months*** after implantation indicate to the disappearing effect of the trauma on the tissues. The variable number of BMP containing osteocytes observed in our study, perhaps, indicate to BMP selectivity depending on biomaterials. *De Jong et al.* (2002) have described, that, despite the study data on the growth factors, including BMP receptors, and BMP as the leading factor of osteoblast differentiation, there is still little known how this factor's biological response and its selectivity are controlled. As to BMP selectivity for calcium phosphate and polymer type activity implants one can find only few studies, in which there are described BMP activity manifestations after HAP and HAP/TCP implantation in the period only up to two months. Thus, *Murata et al*. (2007), two weeks after functionally graded HAP subcutaneous implantation in rats observed intensive degradation of the surface and size of the material and infiltration of giant cells in the biomaterial area, but 4 weeks after implantation there was found the accumulation of albumin containing liquid in the biomaterial, which is explained by the authors as BMP-2/4 intensive effect on the new bone formation process. It seems interesting, that in the study of early postimplantation period the high BMP-2/4 activity is being observed already for two weeks after HAP implantation into the rabbit's mandible (*Salma et al*., 2009). *Ruhé et al.* (2009) had observed that two and four weeks after implantation of BMP saturated calcium phosphate ceramics into the rabbit's skull, there was seen the more intensive formation of the bone structure, in comparison to the control areas, and they pointed to BMP essential role in the bone tissue regeneration process. *Also Yang et al.* (2012) had observed that 4 and 8 weeks after implantation of biphasic HAP/TCP material saturated with recombinant human BMP into the rats' femur, there

have occurred tissue regenerative changes – a new bone formation, however, a comparatively more expressed in the experiment tissues after 8 weeks. *Liporace et al.* (2015), in their turn, by implanting analogous material into rats, had observed an increased bone density both after 4, and 8 weeks. Both *Yang et al.* (2012), as well as *Liporace et al.* (2015) had concluded that HAP/TCP could be used like an effective system for osteoconductive protein delivery and osteocyte regeneration.

In general, the above mentioned BMP-2/4 markers morphological findings in our study can be explained with intensive tissue regeneration, which most actively is occurring during ***3 months***, decreasing after ***4.5 months***, but is still going on for ***6*** and ***8 months*** after the biomaterial implantation, and which is, perhaps, selectively better, when using with PCL coated HAP, β-TCP and unsintered HAP.

4.2.2. Osteoprotegerin (OPG)

As to the number the OPG containing osteocytes were the second most commonly observed cells in all experiment bone tissues, though their number was variable. Thus, ***3 months*** after implantation we found out OPG expressing osteocytes of greater prevalence in the area of α-TCP cement I and in PCL coated HAP tablet, but their number was lesser in the area of sintered HAP-1 and HAP-2 granules, uncoated HAP tablets, HAP/β-TCP-2, β-TCP. ***4.5 months*** after implantation, the number of OPG containing osteocytes was comparatively small, both in the experiment tissues with a different pH α-TCP, and in control tissues. After ***6 months***, though, OPG expression in the biomaterial region had completely disappeared, but after ***8 months*** in the implanted area there was identified the renewal of OPG expression. We did not find any statistically significant differences in OPG release in the experiment and control tissues, but we observed a statistically significant correlation of

OPG and IL-6 containing structures in control tissues ***3 months*** after implantation. The finding mentioned in our study identifies that osteocytes after ***3, 4.5 months, 6 and 8 months*** after calcium phosphate type and polymers-containing biomaterial implantation have a different activity in implantation and also control tissues. Therefore we can admit, that different implantation materials possess a selective property to initiate cell activity and the bone resorption despite the posimplantation time. Here belong such materials as monophasic and biphasic HAP and TCP materials. OPG as a marker of bone tissue activity is described and evaluated also in the studies of other authors. It has been proved in the study of *Nakagawa et al.* (1998) that OPG is a receptor, which can attach to RANKL, to inhibit RANKL and the mutual interaction of the receptor RANK contained in preosteoclasts and osteoclasts. *Lacey et al.* (1998) assert that this receptor is mainly responsible for the osteoclast formation and activity. *Crotti et al.* (2004)

have also mentioned, that RANKL, as well as receptor RANK and their inhibitor OPG are considered for the leading regulators in osteoclast formation both in the healthy, and pathologically changed bone tissues. In the literature almost no studies can be found any studies on OPG expression at certain implantation periods, there are only few data on the observations of OPG expression after calcium phosphate and polymer type biomaterial implantation, among them also in the cases of their losses (it was not found in our study). For instance, *Jiranek et al.* (1993), *Goodman et al.* (1998), *Haynes et al.* (2001) have observed increased OPG and RANKL expression and activity in the cases of lost implants and periimplanted tissues in osteolytic zones (in tissues around the implant). Besides, *Jiranek et al.* (1993) and *Goodman et al.* (1998) have pointed, that also TNF alfa and IL-1 beta regulate OPG and RANKL expression and activity. *Arikan et al.* (2008), determining OPG expression in periimplanted tissues in patients with the root-type dental implants, have investigated, that OPG might be a marker in the assessment of all physiologically morphological

area around the implants. In the studies of other authors on the bone tissue activity associated protein OPG expression using different type biomaterials, among them calcium phosphate and synthetic, and there have been given different data, and OPG expression has been observed both in the implants, and control tissue areas. For instance, in *Crotti et al*. (2004) study on people with joint implants, it was noticed, that OPG expression very little differs in periimplant zones in patients with osteolysis, in synovial tissues in patients with osteoarthritically changed joints, and in health, visually unchanged tissues. *Endres et al.* (2006), using titanium implants of different surface porosity, have observed that all implants promote the bone associated protein – OPG, OC and alkaline phosphotases – expression, but it was essentially expressed in tissues around implants, coated by HAP. It should be added, that in our study there was found a high OPG expression as well in the areas of HAP material implantation. Perhaps, the extra initiator for cell activity, observed in our study, was the inflammation, being proved by a comparatively high, but statistically significantly uncorrelated between themselves both cytokins, and OPG expression in bone tissues of an identical biomaterial, though one cannot exclude an individual response reaction as to the implanted material either. The connection of OPG expression with the inflammation has been described also by other authors. Thus, *Wang et al.* (2015) had observed in patients with periimplant an elevated, though not statistically significant both cytokine IL-1, and OPG expression which, in its turn, was not found in the control group patients. The author thinks that an elevated expression of markers is directly associated with the inflammatory process. *Crotti et al.* (2004; 2015) has also described the association of OPG with the inflammation, which explains the regulation influence of proinflammatory cytokins on the osteoclast activity, which, in its turn, is going on due to the elevated expression of OPG similar NFkappaB ligand receptor activator (RANKL). *Yun et al.* (1998), *Theodore et al*. (2001), *Holding et al.* (2006), *Jiang et al*. (2015) have also indicated to

RANK-RANKL-OPG system activation in inflammatory diseases of the bone-articulation system and periimplantitis. We might think that in the cases of biomaterial implantation, there may be an individual response reaction in the factor release, which has been observed in single cases with experimental animals.

4.2.3. Osteopontin (OP)

In our experiment animals after different biomaterial implantation were observed variable, mainly elevated, bone matrix protein OP expressing cell count in all experiment samples with calcium phosphate (β-TCP and HAP) and polymer type implants and in control samples ***3 months*** after implantation. At the same time such cells were found in the immediate HAP area, and with PCL coated HAP tablets in the implantation area. The before-mentioned points to the possible trauma and material selectivity effect combination, when the trauma decreases the bone cell mineralisation ability, which is preserved only closely to HAP (natural tissue analog), and selectively in calcium phosphate (β-TCP) and polymer implants. It is interesting, that in our experimental animals, ***3 months*** after implantation, in the bone tissues around the implant with a great number of OP containing cells, there was also found a great number of IL-6 cells statistically significantly correlating with it, pointing to the hidden activation of other cytokins (*Feurino et al.,* 2007). It is affirmed also by *Zhang et al.* (2014), stating that IL-6 is the inflammatory I phase cytokine.

In our animals, ***4.5 months*** after α-TCP implantation, OP containing bone cells were not found. But ***6 months*** after implantation OP factors showed a moderate amount of the control cells, but after ***8 months*** OP was found also in experiment bone tissues. Although ***3, 6 and 8 months*** after implantation, the average number of OP containing cells in the experiment and control groups did not differ, the data mentioned, to our mind, reveal an interesting tendency in

OP release, i.e., ***4.5 months*** after implantation it is the possible time when cells are the least active (it is also justified by the smallest findings of OPG, and the lack of different, as well as cytokin and antimicrobial protective proteins in cells just at this period), while after ***6 months*** the effect of trauma on supportive tissues is practically gone, but ***8 months*** after implantation it is the time when supportive tissues are restoring their functional activity and antimicrobial protection. It is important to mention here, that in the data analysis, ***3 months*** after implantation we found in the experiment group a close, statistically significant correlation between **OP** and **OC, βDef-2, IL-6 and IL-10 release,** but in the control tissues between **OP and IL-1, IL-8 and βDef-2** release. And only after ***8 months*** we observed a close statistically significant correlation between **OP and βDef-2** release. All these changes correlate with the close effect of biomaterial on tissues, the best of which is for HAP and its analogs. In bone tissues *Nanci* (1999) and *Launey et al*. (2010) have found OP in the extracellular matrix between mineralized collagen fibres. *Fantner et al*. (2005) has revealed an interesting fact that OP, like a noncollagenous factor is acting also as an adhesive material in the bone tissues. *Sodek et al*. (2000) indicate to the importance of OP and the differences of expression in the bone tissue development, regeneration, resorption and calcification processes, mentioning that OP has an essential significance in the process of osteoclast formation, migration and cell resorptive activity. *Scatena et al*. (2007) consider that OP, however, inhibit the mineralisation, protecting the body from ectopic bone tissue formation. *Sodek et al.* (2000) mention also OP proinflammatory properties, due to which it is involved into the immune system response reaction. OP appears to stimulate the immune cell reaction to release IL-1, IL-6 and TNF, but to inhibit apoptosis. *Choi et al.* (2008), however, have found the highest OP level in blood serum in ankylyzing spondylitis patients, saying, that OP prevailing role is in the bone remodeling process, leaving the importance of this factor as secondary in the maintenance of the inflammation.

Double functions – OP as the phosphorilation-inducing factor involvement into the biomineralisation for bone tissue remodeling, and for the maintenance of the immune system response reaction – are indicated also by *Li et al.* (2015). Not neglecting both of these functions, which evidently do exist also in our study, we want to emphasize, that we agree also to the point of view of *Hunter* (2013) and *Holm et al*. (2014), that the inhibiting or stimulating effect of OP like a component of the extracellular matrix of mineralized structures on the formation of the mineral – hydroxyapatite is still unclear. No selective effect of different biomaterials on OP release is mentioned because no extensive research was carried out, however, it is possible to look through our results in the context of solitary studies. Our study data coincide with single studies (*Lee et al*., 2011) on the stimulating effect of OP expression in cells in calcium phosphate type biomaterials. *Lee et al*., (2011) describe also an interesting observation, that titanium implants coated by HAP causes both OP and OC increase in the implantation areas already ***3 months*** after implantation.

Just the HAP was found to be an essential biomaterial component, besides, it is not important how great HAP prevalence is in the biomaterial (*Ruckh et al*., 2012), because already 3 weeks after implantation of different concentration (1% and 10%) of HAP and PCL composite one can find vast OP nests. In such a way, our findings on OP containing cells just nearby to HAP are essential and confirm *Zheng et al*. (2014) statement that HAP and OP possess high interactive potential. We can admit therefore, that in all our experiments, where we were analyzing OP, the following essential factors were identified: 1) initial trauma as OP depression-reducing factor; 2) HAP-containing material, as well as calcium phosphate and polymer implants like selective OP expression-provoking factors and 3) the essential ***3 and 8 months*** after implantation, of which the first time is associated also with tissue response reaction on the implantation trauma, but the second one indicates to a complete restoration of the bone tissue factor expression.

4.2.4. Osteocalcin (OC)

After some of biomaterial implantations our experiment animals were observed to have also a variable number of bone matrix protein OC containing cells, mainly ***3 months*** after HAP-1 granules, with PCL covered HAP tablets, β-TCP-2 and uncommercial PMMA cement implantation. With the implantation time growing from ***4.5 months*** till ***8 months***, OC containing cells were found to be in a lesser amount. In the data analysis we did not observe any difference in the average number of OC containing cells, but their increase after ***3 months*** statistically significantly correlated with OP and βDef-2 expressing cell count, but ***4.5 months*** after implantation the number of OC expressing cell count statistically significantly correlated with IL-1 and βDef-2 expressing cell count.

The findings mentioned point to a more active bone tissue mineralization ***3 months*** after implantation, which, perhaps, deals with a selective biomaterial effect on bone tissue mineralization. Such an effect had been observed in the analysis of bone matrix protein OP expression, where we saw a more marked OP expression ***3 months*** after calcium phosphate and polymer implantation. The above-mentioned still does not also exclude a possible combination of trauma and material's selectivity influence, when trauma decreases the ability of bone tissue mineralization, being preserved only in HAP area and selectively in the areas of calcium phosphate and polymer implants. Along with the bone matrix protein findings, in our study it is essential to mention also the functions and the role of OC in other authors' studies. For instance, *Lee* (2000) and *Lombardi et al*. (2015) have described that OC can be found in osteoblasts, odontoblasts, cementoblasts and chondroblasts. *Davis* (2006), *Lee* (2007) and *Lombardi et al*. (2015) have indicated to the intensive involvement of OC into bone metabolism and osteogenesis. To our mind, it is important to mention here also *Robey et al*.

(1993), *Young* (2003), *Hamada et al.* (2008) observations, that bone matrix proteins – OP and OC – have the main role in the collagen I integration process with HAP crystals in the bone matrix. A comparatively more marked OP and OC expression in our experiment's bone tissue samples and just the findings in HAP and calcium phosphate implant areas in our study point to these materials' selective influence on OP and OC expression, grounded by *Robey et al*. (1993), *Young* (2003), *Hamada et al.* (2008) observations. No selective different biomaterial influence also on OC release is mentioned in the literature, because no wide research has been done on implantation of calcium phosphate and polymer type biomaterials in relation to the bone matrix protein expression. However, despite it, one can look through our study results in the context with some other authors' research, which still indirectly justify and depict the stimulating effect of HAP implant on OC expression as well. *Endres et al.* (2006), using titanium implants with a different surface porosity, observed that all implants promoted the expression of bone associated proteins – OPG, OC and alkaline phosphatase, but more expressed it was in the tissues around the implants, coated by HAP. *Santarelli et al.* (2014), studying biocompatibility in vitro of the natural, from a bovine-acquired HAP and synthetized nanocrystal hydroxyapatite, using MG-63 cell line (a human osteosarcoma cell line), found variable osteogene markers OC and OP expression in the experiment and control tissues. In our study we observed a stimulating effect of polymer-type implant PMMA on OC expression, which has been described in other authors' studies as well. *Zambonin et al*. (1998) in human osteoblast culture in vitro study observed an elevated OC and IL-6 expression in PMMA powder-type particles in implantations areas and at the same time marked inhibited osteoblast proliferation and collagen synthesis. He also indicated that PMMA particles, influencing the osteoblast activity, together with other factors can produce periprosthetic osteolysis (osteolysis in tissues around the articulation implants) in the following ways: 1) decreased osteoblast proliferation and

collagen synthesis slow down the bone formation; 2) to osteoblast inhibition related activation of osteoclastic bone resorption proceeds along with the increased OC and IL-6 synthesis. *Zambonin et al.* (1998) has also concluded that OC can promote accumulation of osteoclasts on the bone surface and IL-6 – to cause osteoclastogenesis. The relation of IL-6 to osteoclastogenesis in many authors' studies is being explained by its regulating influence on osteoclasts activity as a result of elevated expression of OPG similar NFkappaB ligand receptors activator (RANKL) (*Holt et al.,* 2007; *Polzer et al.,* 2010; *Crotti et al.* 2015). But an increased release of IL-6 in implantation cases is related to implants' own release of activated macrophages IL-1 and TNF-α (*Schmidt et al*., 2003). *Ohsawa et al*. (2001) after PMMA particle implantation into the rat's shin-bone have also observed the stimulating effect of PMMA on OC expression, which was going on simultaneously with osteonectin expression. In our study, however, a comparatively great number of OC expressing cells was seen ***3 months*** after implantation also in monophasic β-TCP and biphasic HAP/β-TCP regions, though the number of IL-6 expressed cells was rather small, which may probably indicate to IL-6 still not participating in osteoclastogenesis. Taking into account, that both the number of OC expressing cells, and also the number of IL-6 expressing cells were observed ***3 months*** after commercial and noncommercial PMMA cement and with PCL coated HAP tablet implantation, the mentioned findings in our study show that osteoclastogenesis is proceeding selectively. In our experiments, where we analyzed OP and OC, we marked the common, essential features for these markers: the initial trauma influences both OP, and OC expression; HAP containing material, as well as calcium phosphate and polymer implants are selective OP and OC expression-inducing factors; a more pronounced OP and OC expression ***3 months*** after implantation is associated also with the tissue response reaction on the implantation trauma, but at the later time – ***8 months***

after implantation – simultaneously point to the restoration of expression both in OP, and OC.

4.2.5. Proinflammatory (inflammation inducing) cytokins interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8)

In our study the expression of proinflammatory cytokins IL-1, IL-6 and IL-8 was observed in all post-implantation months both in the experiment, and in control tissues, except for ***4.5 months*** after implantation. In its turn, the difference in the statistically significant cell count was found only in IL-1 ***3 months*** after implantation when there were also seen more IL-1 positive osteocytes after implantation of α-TCP cement I, but IL-6 – after commercial PMMA cement (also IL-8 containing cells), α-TCP cement I and with PCL coated HAP tablet. Besides, in the analysis of the mutual correlation of these factors we did not acquire any statistically significant correlation in any of postimplantation periods either in the experiment, or control tissues.

Taking into account the mentioned findings, we consider that the most pronounced (objectively – IL-1, subjectively – also IL-6 and IL-8) cell number in the first ***3 months*** points to a hidden inflammation as a response reaction both on the tissue traumatization, as well as, perhaps, on the biomaterial, which subsides in a later period of time. Tissue traumatization-related inflammation has been reviewed also in other authors' studies, but, in general, it is known, that in the place of the inflammation the cytokines, released by infiltrating macrophages, especially IL-1, possess a decisive role in the pathogenesis of the local inflammation and tissue damage, which can be explained by IL-1 stimulating effect on adhesive molecule expression from endotheliocytes (*Dinarello*, 1988, 1994; *Tracey*, 1989). At the same time, cytokine IL-6 stimulates the release of other cytokines (*Feurino et al*., 2007), and as described

by *Zhang et al.* (2014), it is acting as the inflammation-stimulating cytokine, and is being the leading cytokine in the initial phase of the inflammation where the inflammation is caused by trauma, which, to our mind, would be attributable also to biomaterial implantation. IL-6 is secreted by T lymphocytes and activated macrophages in the blood-vessel wall, thus stimulating the immune system response reaction to trauma, burns and other tissue damages, proceeding with the inflammatory reaction (*Ostriker*, 2014). Cytokine IL-8 as chemokine protein (*Oppenheim et al*., 1989; *Baggiolini et al*., 1992) of the inflammation-facilitating activity and the characteristic effect of haemotaxis function is one of the main cytokines which, in the case of inflammation, activates neutrophil leukocytes (*Bickel*, 1993; *Rosenthal et al*., 1994; *Gilmour et al*., 2003) and provides the body response reaction to the damage. At the same time, *Qazi et al*. (2011) has described the interrelation of IL-1 and IL-8 effect and have indicated, that IL-8 synthesis is closely connected with IL-1 and TNF-α stimulating effect. In the study of morphological changes it is important to assess proinflammatory cytokines IL-1 and IL-6 expression, because there is found evidence in the literature on IL-1 and IL-6 possessing osteoclastogenesis-facilitating properties with the subsequent bone tissue resorption (*Holt et al*., 2007; *Polzer et al*., 2010) and loss of implant fixation (*Holt*, 2007). It is also known, that implants' own activated macrophages release IL-1 and TNF-α, which, in its turn, stimulates osteoblasts and can promote IL-6 and prostaglandin E-2 secretion (*Schmidt et al*., 2003). And so, altogether there are activated also osteoclasts with the subsequent bone resorption and loss of implant fixation. In our study we did not notice any signs of implant loss in animals in neither of any implantation periods, although after ***3 months*** we found comparatively many both IL-6, and OC positive osteocytes. Numerous IL-6 positive cells and at the same time a abundance of OC positive cells were found in HAP-1 implantation region, but in monophasic β-TCP region – moderate number of IL-6 containing cells, and abundance of OC cells.

Besides, also ***3 months*** after implantation of PMMA cement and with PCL coated HAP tablets there were found an abundance IL-6 containing cells. Evidently, the mentioned findings in our study can be explained with the less aggressive and selective effect of the biomaterial on the expression of these biomarkers and the resulting consequences.

And yet, there are very few studies described in the literature which are similar to our study, just refering to this, before-mentioned factor expression from the bone implant regions. And so, *Ninomiya et al.* (2001) states, that HAP and biphasic HAP/β-TCP stimulate the bone IL-6 and protease expression, in such a way increasing the bone resorption, osteolysis and the ability to lose the implant. *Eyckmans et al*. (2013) in his turn, states a marked osteoblast IL-6 and BMP expression in Ca-P implantation case. The mentioned study data correspond to our findings as to a comparatively great IL-1 and IL-6 containing cell number in the regions of calcium phosphate containing biomaterials, giving the evidence of a hidden inflammatory process, as well as of IL-6 stimulated other cytokines release (*Feurino et al*., 2007). We are, however, of the mind, that IL-1 and IL-6 release only points to the inflammation without any visual inflammatory cell findings, which, within the period time, reduce. *Schminke et al*. (2015) have described the observation that in 30% implantation cases, periimplatitis in patients develops as a serious postimplantation complication, causing loss of supportive tissues. The authors have marked, that in such a case, there increases IL-8 expression. *Konttinen et al.* (2006), in their turn, have found a different reinforced cytokine – IL-1 and IL-6 – release and osteoclasts activation in periimplantitis and chronic periodontitis patients. *Svenson et al*. (2015) have found in the tissues an elevated IL-6, IL-8 and TNF-α expression, the latter being as a biomaterial-associated infection, where titanium surface was infected by *Staphylococcus aureus.* In our experiments, ***3 months*** after implantation we observed a less pronounced inflammation only in the commercial PMMA cement area alongside the elevated IL-8 expression,

besides the cytokine-containing cell number in this case did not significantly differ from the cell number in tissues with other implants.

The evidence can be found in the literature, that the biomaterial content significantly affects the release of these cytokines, if the surface coating is similar (*Schmidt et al*., 2003). *De Wilde et al.* (2015) had not found any statistically significant differences in IL-1, IL-6 un IL-8 expression in soft and hard tissues 8 weeks after pure and nano-hydroxyapatite-coated titanium implantation, pointing that biomaterials with HAP coating might be more biocompatible. In our study the greatest IL-1 inductor was α-TCP cement I and IL-6 inductor – commercial PMMA cement, α-TCP cement I and by PCL coated HAP, which substantiates our, the already mentioned, hidden inflammation process.

In general, we have to say, that the findings of our study in proinflammatory cytokine expression – the decrease of IL-1 containing cell number for ***4.5 months*** and in a longer period of time after implantation, a complete IL-6 and IL-8 containing cell number depletion for ***4.5 months*** after implantation, but its restoration ***6*** and ***8 months*** after implantation can be explained by the decrease of the effect of the implantation trauma, selective body response reaction on trauma and biomaterial. This fact also indicates the importance of IL-6 and IL-8 for a longer regeneration process. It is interesting to mention tricalcium phosphate material, i.e., already ***3 months*** after implantation in the area of β-tricalcium phosphate material there was observed variable IL-6 expression and a comparatively less pronounced IL-8 expression too. In its turn, ***4.5 months*** after implantation there was seen the decrease of IL-6 and IL-8 containing cell number and depletion, which, perhaps, point to selective influence of tricalcium phosphate in cytokine expression. Taking into account the fact, that in our study after ***4.5 months*** there was found the decrease and even depletion of OP expression, these findings should be associated with the interrelation of other authors' already identified OP and proinflammatory

cytokine expression (*Sodek et al*., 2000), where the authors had noted the OP stimulating effect on the immune cells' capacity to release IL-1 and IL-6. It is interesting also to mention the observations by *Podaroppoulos et al*. (2009) about the less pronounced new bone formation 4 months after β-tricalcium phosphate implantation, where the authors speak about the effect of ambiguous, β-tricalcium phosphate containing, calcium ions on the bone tissue regeneration. The above-mentioned is confirmed by observations, that on one hand, during biomaterials resorption, the increased liberated calcium ions can stimulate the osteoclast activity (*Yamada et al*., 1997), but on the other hand – it provides a good microenvironment for osteogenesis (*Fujita et al*., 2003).

We still have to mention, that ***4.5 months*** after implantation, it is the time of decrease of cell functional activity, which is justified also by the least OPG findings in our study. On the other hand, on the ***6th*** postimplantation ***month***, the cell functional activity changes, when the effect of trauma on supportive tissues disappears, and ***8 months*** after implantation, it is the time when the functional activity of supportive tissues after implantation process is restored, which is proved by restoration of OPG, OP and OC expression activity. In parallel to it, restoration (though less pronounced) of proinflammatory cytokine and antimicrobial defense protein release is goin on. The above-mentioned is grounded by the identified correlations of these factors, i.e., the close statistically significant correlation between OP and βDef-2 release, and between OP and IL-6 release in the experiment group and IL-1, IL-8 and βDef-2 release in control tissues ***3 months*** after implantation, but ***8 months*** after implantation between OP and βDef-2 release in control tissues.

4.2.6. Antiinflammatory (antiinflammatory) cytokine interleukin-10 (IL-10)

IL-10 expression was found in all postimplantation periods, except after ***4.5 months*** in both – the experiment, and control tissues, and the cell number of these factors ***3, 6 and 8 months*** after implantation did not statistically significantly differ. The data correlation analysis, in its turn, showed statistically significant close correlation between the antiinflammatory cytokine IL-10 and proinflammatory cytokine IL-1 release, as well as IL-10, βDef-2 and IL-10 and bone matrix protein OP release in the experiment bone tissues ***3 months*** after implantation. In this postimplantation period the most of IL-10 positive osteocytes were found after implantation of commercial PMMA cement, β-TCP, PCL coated HAP and uncoated HAP tablet. But in control tissues we did not observe IL-10 correlation with other factors. The mentioned findings give evidence of the local response reaction of preserved tissues by trying to inhibit the inflammation, because IL-10 is the 2nd type cytokine, which is considered the antiinflammatory cytokine, and which has a significant role in the regulation of the immune system and the inflammatory process (*Eskdale*, 1997), produced by such immune system cells like T cells (Th2, Th1, Th17) (*McGuirk and Mills*, 2002), monocytes, macrophages, dendritic cells(*Fillatreau et al*., 2008), granulocytes, including eosinophile leukocytes and mast cells(*Ryan et al*., 2007). But the loss of IL-10 ***4.5 months*** after implantation can be explained by the proinflammatory cytokine activity decrease, when IL-1 expression was also less pronounced. The restoration of the observed IL-10 expression ***6 and 8 months*** after implantation could be explained with IL-10, like an immune system's regulator's role, mentioned by *Eskdale* (1997), and which is preserved for a longer period of time – ***6 and 8 months*** after the implantation of biomaterials. Studies are mostly found on the general reactions of the immune system in the implant regions, but not on a

certain cytokine expression of the very bone, therefore our findings are estimated as new and original, which confirms just the role of the bone itself for the weakening of the local inflammation. Our assumption is confirmed by *Numerof et al*. (2006) who described IL-10 anatgonistic influence on the proinflammatory cytokine TNF-α, IL-1, IL-8, IL-12 release and the stimulating influence on B lymphocyte proliferation and antibody production, as well as *Zhou et al.* (2005) study on the pronounced suppressive effect of IL-10 on the production of the inflammatory mediators. Unclear and little studied is the cytokine IL-10 expression in the correlation with the implantation time. Already 2 weeks after implantation of HAP, saturated by dexamethazone and lidocaine, *Salma et al.* (2009) has found the release of this factor, which, perhaps, is connected with the primary inhibiting effect of medicines on the surrounding tissues of the implant. In his turn, *Reinis et al.* (2011) pointed that after two and 4 weeks, after glass-calcium phosphate ceramic implantation in rabbits, there was observed an elevated IL-10 expression around the biomaterial, which was treated by *P. aeruginosa* culture, as well as the fact, that two and four week implantation time did not affect IL-10 expression in the cases of implantation of biomaterials. It is interesting here to mention the study by *Velard et al.* (2013), in which the authors point to the role of biomaterial content in the tissue regeneration, i.e., HAP and β-tricalcium phosphate, possessing high osteoinduction and the fact, that as a result of interaction of these biomaterials and the surrounding tissues there may be induced the immune system's cell response reaction, which can be differently pronounced.

It is also essential to mention the study by *Hoene et al*. (2015), in which 56 days after intramuscular implantation of surface modified titanium into rabbits, the authors observed a statistically significant correlation between the proinflammatory cytokine interferon γ and IL-2 expression and CD-68 positive monocyte infiltration, while the correlation was not observed between the

antiinflammatory cytokine IL-10, IL-4 expression and the inflammatory cell infiltration.

All in all, on the basis of our findings of the relatively homogenous factor reaction, we consider IL-10 being one of the universal longstanding inflammation-inhibiting factors, which disappear during the loss of the cell's functional activity (***4.5 months*** after implantation of the biomaterial) and is relatively selectively induced by different biomaterials.

4.2.7. β defensin-2 (βDef-2)

The greatest number of antimicrobial protein βDef-2 containing osteocytes in the regions of biomaterial implantation was seen ***3 months*** after implantation. The mentioned findings point to the more active inflammatory process in this period of time, which corresponds also to other authors' data on the development of the inflammation around the implants, stating that the process is developing up to 3 months (*Jones et al*., 2001; *Trampuz et al*., 2005; *Higgins et al*., 2009). In our study the greatest number of βDef-2 containing osteocytes was prevalently observed in the area of implantation of uncoated HAP tablet, which evidently prove about the release selectivity of this factor to HAP material in the comparatively early postimplantation period. ***4.5 months*** after α-TCP (with a different pH) implantation, βDef-2 in the tissues was found only in rare βDef-2 containing cell count in α-TCP with pH 8.0 and pH 6.0 region, but ***6 and 8 months*** after implantation with HAP-2 material, βDef-2 positive cells were not noticed at all. The correlation analysis of the factors showed a statistically significant correlation between βDef-2 and the antiinflammatory cytokine IL-10 release, but a statistically significantly moderately close correlation between βDef-2 and proinflammatory cytokine IL-1 release in the experiment tissues ***3 months*** after implantation. The mentioned βDef-2 containing cell findings are related with proinflammatory

and antiinflammatory protein activity just for ***3 months***, and the cell functional depletion ***4.5 months*** after implantation, which means a more active inflammation process in the tissues in the comparatively early postimplantation period. We might think, that βDef-2, proinflammatory and antiinflammatory protein expression decreases with the decrease of the inflammation, when the tissue regeneration process is getting restored as well.

β-defensin' role and functions in the antimicrobial defense system are rather widely described (*Crovella et al*., 2005; *Veerayutthwilai et al*., 2007; *Vordenbäumen et al*., 2010; *Nakatsuji and Gallo*, 2012), however, there are only few, and contraversial data on the release of defensin in the inflammatory-affected tissues, especially in the bone tissues in biomaterial implantation. In the study on patients with periodontitis and periimplantitis (*Bissell et al*., 2004) there has been pointed out that the highest βDef-2 expression was observed just in the healthy adjacent tissues, not in the periodontitis and periimplantitis zones. *Reinis et al*. (2011) two and four weeks after glass-calcium phosphate ceramic implantation in rabbits had observed a more pronounced βDef-2 expression in tissues after two weeks either in the biomaterial area, or control tissues. However, the findings in some recent studies show, that βDef-2 expression most prevalently proceeds in the implantation area, not in the control tissues. For instance, in the study by *Ertugrul et al*. (2014), the patients with periimplantitis have been observed a higher βDef-2 level in periimplant's tissues, which, by the authors, is related to the tissue response reaction on implantation (which was also decisive in our study), and the bone resorption and microbial proliferation in the tissues around the implant. It is interesting to mention also the study by *Warnke et al*. (2013), where by incubation of mesenchymal cells, osteoblasts, keratinocyte cell cultures with βDef-2, there was identified a good biocompatibility, and even the protein's stimulating effect on the mentioned cell proliferative activity. The authors, however, point, that these findings are not convincing and it would be worth doing further

studies on the proliferative activity of different cells under the influence of defensins on the functional surface of dental implants.

In general, estimating the findings in our study, we should mention that the initially pronounced release in the bone tissues of the main antimicrobial protein βDef-2 ***3 months*** after implantation correlates with the inflammation process and, alongside with its disappearance it starts slowly restorings on the ***6th and 8th*** postimplantation ***months*** and only in control tissue regions. This fact and the loss of the influence of biomaterials prove that primarily in the bone tissues there are prevented the inflammation and posttraumatic changes, followed by a slow restoration of antimicrobial protein synthesis. In general, these findings are estimated as new and essential in experimental implantology.

4.2.8. Apoptosis findings

Apoptotic cells were identified in all postimplantation periods, but more prevalent in the early postimplantation time **– *3 months*** after implantation they were more prevalent in the areas of unsintered HAP and sintered HAP-1, β-tricalcium phosphate and polymer-type implants. Within the time mentioned, there were found also a more pronounced antimicrobial protein, proinflammatory and antiinflammatory cytokine expression, of which just the proinflammatory cytokine expression was likely to be closely related with the increase of apoptosis, which is seen also by statistically significantly greater number of apoptotic osteocytes and of proinflammatory cytokine IL-1 containing osteocytes in the area of implants within this period of time. Our findings coincide with other authors' studies which explain both apoptosis and the inflammatory mutually independent process, and cell apoptosis due to the inflammation, as well as apoptosis can even indirectly inhibit the development of apoptosis. For instance, *Fadok et al*. (1998) un *Savil et al*. (2000) have described that in apoptosis or programmed cell death way, the processing of

cell components is provided both by macrophages, and dendritic cells, but there are not released the intracellular components, in such a way not causing the inflammation within the time of the apoptosis process itself. Here it is also interesting to mention the assumption of *Fadok et al.* (1998) and *Savil et al.* (2000) that receptor and opsonin attachment to the apoptotic cell ligands provides their recognition and modified phagocytosis and in such a way there is inhibited the release of the inflammation mediators and the increase of TGF-β1 production. Also *Peng et al.* (2007) confirms the mentioned assumption, explaining that the dendritic cells, getting into contact with the apoptotic cells, inhibit the cytokine release and they do not mature, thus the development of the inflammation is not promoted. *Gamonal et al.* (2001), in their turn, have described that in the case of chronic inflammation the apoptotic process gets activated and the number of apoptotic cells increases which resembles the findings of ***3 months*** after implantation in our study. It is interesting to mention several studies on the effect of biomaterials on apoptosis, and these findings coincide with those observed in our study respectively, HAP is facilitating the cell apoptosis. For instance, *Salma et al.* (2009) two weeks after unsaturated and lidocaine- saturated HAP implantation had observed a comparatively numerous apoptotic cells and also TGF-β1 containing osteocytes in the experiment tissues, but evenly numerous IL-10 positive cells both in the experiment, and control tissues. *Shi et al.* (2009) in the human osteoblasts in vitro had observed that HAP particles facilitate apoptosis and its intensity depends on their size, but regardless of it, this material has a good biocompatibility. Also *Xu et al.* (2012) in the study on different sizes, forms and surface of HAP particles had found in the rats' osteoblasts that HAP inhibits the mentioned cell activity, but at the same time facilitates the cell apoptosis, the intensity of which is, in its turn, dependant on the size and form of these particles. At the same time we can find the data, saying, that the connection of apoptosis development with the type of the biomaterial in an

early implantation period is not convincing. For instance, *Bombonato-Prado et al*. (2009), in the study of PMMA and HAP material, using the human osteoblast culture, had not found changes after 7 days in the apoptosis regulating gene analysis.

In the later postimplantation periods of our study – ***4.5, 6 and 8 months*** after implantation – there was identified the gradual decrease of apoptotic cell number from moderately extent apoptotic cells in the region of α-tricalcium phosphate with different pH up to few apoptotic cells after ***6 un 8 months*** in HAP region. The mentioned findings could be related to the decrease of antimicrobial protein, proinflammatory and anti-inflammatory protein expression ***4.5 and 8 months*** after implantation, identifying the mutual correlation of the inflammation and the apoptotic process. The less pronounced apoptotic process in our study after ***4.5 months*** could be related also to the changes of the cell functional activity (its decrease), which is justified also by the decrease of OPG release in our study. But after ***6 months*** the functional activity of cells changes, due to the loss of the effect of trauma on the supportive tissues, and ***8 months*** after implantation is the time when the functional activity of supportive tissues after the implantation process is renewed, the evidence of which is the restoration of OPG, OP and OC expression activity. In the literature, in fact, there cannot be found any studies on the biomaterials used in our experiment, and relationship of apoptosis in these implantation periods of time, but as to the relationship with the 4^{th} and 6^{th} months' postimplantation time it is worth to mention the observations in the eye tissues of *Ibares-Frías et al*. (2015) study, in which the authors describe the active apoptosis in the implantation period up to 72 hours, but in a later period, including the period after 1, 3, 4 and 6 months, they had observed only single apoptotic cells, which, by the authors, are connected with the inflammation and more active tissue regeneration in the early postimplantation periods.

In the literature one can find interesting data on the relationship of OP, inflammatory cytokine and apoptotic process. OP appears to stimulate the ability of the immune cells to release IL-1, IL-6 and TNF, but to inhibit apoptosis (*Sodek et al*., 2000). In our study the inhibiting effect of OP was not observed, just the opposite – in ***3 months***' period, when an elevated OP expression was observed, there was a comparatively great, though a variable number of apoptotic cells. Evidently, it is significant here to have the selectivity combination of the effect of the inflammation and the biomaterial, which in our case is attributable both to the pure HAP material, and tricalcium phosphate and polymer-type implants.

The second important factor in the cases of the biomaterial implantation is the traumatic damage. *Web et al*. (1997) and *Ratner et al.* (2013) have indicated that the biomaterial implantation process is connected with the direct mechanical action on the tissues, which is the one of the causes of the development of the apoptotic process. In the study of cochlear implants *Bas et al*. (2012) relate the cell apoptosis to the inflammation due to the effect of implantation trauma and the oxidative stress. Also *Jia et al*. (2013) in the review analysis on the effect of implantation trauma on apoptosis have concluded that the apoptotic process is connected with the trauma and the inflammation resulting from it, which was confirmed by the correlation of proinflammatory cytokine expression and the apoptotic process in the postimplantation period.

It might be that the mentioned correlation is attributable also to our study, in which there were identified changes of biomarkers – a high antimicrobial protein, proinflammatory and anti-inflammatory cytokine expression and the apoptotic cell death – all this is connected with the cell traumatic damage during the implantation of biomaterials, which was actively exposed just during ***3 months*** after implantation. One cannot exclude the apoptosis due to biomaterial as a chemical substrate, because in our study we

had observed a selective apoptotic process (different apoptotic cell count) both in “pure” HAP material, and tricalcium phosphate and polymer-type implant areas. Also *Kokesch-Himmelreich et al*. (2013) in the study of calcium phosphate have observed that the biomaterial content is affecting the cell apoptosis, i.e., strontium coated calcium phosphate cement more actively than the “pure” cement inhibits apoptosis and at the same time promotes also the osteoblast differentiation. In general, apoptotic process in our study proves that apoptosis in the case of biomaterial implantation proceeds under the influence of the inflammation due to traumatic damage and biomaterial selectivity combination.

5. CONCLUSIONS

1. Biomaterial implantation in soft tissues causes nonspecific local tissue response reaction on trauma/implantation, characterized by the tissue edema, lymphocyte infiltration, sporadic nidus-type granulations and fibrosis, despite the type of the implant material. A new bone formation in the supportive tissues occurs in the 3rd implantation months after the implantation of uncoated HAP tablet, PCL coated tablet, PMMA cement and unsintered HAP granule implantation without extensive bone resorption zones, which indicates to the greater osteoinductivity of the mentioned material in comparison to different tricalcium phosphate cements (sintered β-TCP at different temperatures, with a different pH monophasic α-TCP and biphasic HAP/β-TCP) and sintered HAP granules.
2. BMP-2/4 as the bone morphogenetic protein is a constant bone growth factor with common tendency to increase in the bone tissues from the 3rd postimplantation months and this tendency remains during 6 and 8 months after implantation. BMP-2/4 expression is selectively dependent on the type of the biomaterial, while the slow restoration of the release of factor in the control side identifies the trauma effect on the tissues healing.
3. Different OPG expression points to the selective bone tissue resorption stimulation. The bone resorption proceeds simultaneously with the inflammatory process, identified by a close correlation of OPG release with IL-6 release 3 months after implantation.
4. Three months after implantation of different biomaterials the elevated OP cell number alongside the elevated IL-6 cells points to the hidden trauma and biomaterial caused inflammation. 4.5 months after implantation is the time of decrease of the cell functional activity, which is justified by the smallest findings of OPG and the lack of cytokines, which change in the 6th postimplantation months, with the loss of trauma effect on the supportive

tissues. Eight months after implantation the supportive tissues restore their functional activity.

5. The most pronounced number of bone tissue matrix protein OC containing cells 3 months after the implantation of sintered HAP granules, PCL coated HAP, β-TCP-2 and uncommercial PMMA identifies a selective stimulation of the bone tissue mineralization just by these biomaterials.
6. The most pronounced number of proinflammatory cytokine – IL-1, IL-6 and IL-8 expression 3 months after implantation of different biomaterials justifies the tissue response reaction both on the trauma, and on the biomaterial, causing the cell functional depletion 4.5 months after the implantation, though later getting restored, thus confirming the gradual disappearance of hidden inflammation.
7. Antiinflammatory cytokine IL-10 is characterized by a constant release in the bone tissues after implantation of different biomaterials, selectively after the implantation of commercial PMMA cement. The greatest findings of IL-10 containing cells 3 months after implantation show that at this time there is most actively inhibited the biomaterial-caused inflammation, but the lack of IL-10 containing cells after 4.5 months prove that the local cell defense system has been exhausted.
8. The main, initially pronounced release of the antimicrobial protein βDef-2 in bone tissues of the body during 3 months after implantation correlates with the inflammation process and the release of proinflammatory and anti-inflammatory cytokines, and, with the disappearance of the inflammation, it starts restoring after 6 and 8 months only in the control side. These findings, in combination with the lack of selective effect of biomaterials prove about the prevention of primary inflammation/traumatically–caused changes occurring in the bone tissues with a subsequent restoration of slow antimicrobial protein release.

9. Apoptosis in the case of biomaterial implantation occurs as a result of the inflammation due to traumatic damage and selective combination of biomaterials, which are more actively exposed 3 months after implantation.
10. In the morphological determination of the tissue reactogenicity/biocompatibility 3 months after implantation the most essential is BMP-2/4 for HAP and its containing materials, and proinflammatory cytokine IL-1 for calcium phosphate and polymer materials, which is proved by a statistically significant release of these factors in the bone tissues of the experiment; due to a scarcely pronounced release of factors 4.5 months after implantation, this postimplantation period would not be included into the diagnostic algorithm; in the HAP implantation case, after 6 months, there should be identified the proinflammatory cytokine IL-8 and the bone matrix protein OC, but after 8 months – the proinflammatory cytokine IL-6, antiinflammatory cytokine IL-10 and the bone cell activity marker OPG, because in the mentioned periods of time the release of these factors had got restored within the normal range.

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7. PUBLICATIONS AND PRESENTATIONS ABOUT THE STUDY

Scientific publications

1. **Vamze J**., Pilmane M., Skagers A. Biocompatibility of pure and mixed hydroxyapatite and α-tricalcium phosphate implanted in rabbit bone // J Mater Sci: Mater Med, 2015; 26:73. doi: 10.1007/s10856-015-5406-6.
2. **Vamze J**., Pilmane M., Skaģers A., Šalms Ģ., Irbe Z. Kaula morfoģenētiskā proteīna, osteoprotegerīna, osteopontīna, osteokalcīna ekspresija truša apakšžokļa un stilba kaulaudos pēc dažādu biokeramikas materiālu implantācijas // RSU Zinātnisko rakstu krājums, 2012; 119–128.
3. **Vamze J**., Pilmane M., Skagers A. Activity of host defense proteins in rabbit bone after pure hydroxyapatite and tricalcium phosphate and mixed tricalcium phosphate/hydroxyapatite implantation // IFMBE Proceedings, 2012; 38: 110–112.
4. **Vamze J**., Pilmane M., Skagers A. Cytokine and HBD-2, -3, -4 expression in rabbit bone tissue after hydroxyapatite (Hap), α-tricalcium phosphate (α-TCP) and polymethylmetacrylate (PMMA) implantation // FMNT issue of IOP conference series: Material Sciences and Engineering, 2012; 38: 012025. doi: 10.1088/1757-899X/38/1/012025.
5. **Vamze** J., Pilmane M., Skaģers A. Kaulaudu reģeneratīvo procesu noteicošo proteīnu izmaiņas truša apakšžokļa kaulā pēc HAp (hidroksiapatīta) implanta // RSU Zinātnisko rakstu krājums, 2011; 167–174.

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1. **Vamze-Liepina J**., Pilmane M., Skagers A., Salms G., Loca D. Characteristic of osteopontin, osteocalcin and osteoprotegerin expression in rabbit bone tissue after the implantation of hydroxyapatite-containing

biomaterials [poster presentation] // Abstract book: p. 191; International Conference on Functional Materials and Nanotechnologies, October 5–8, 2015, Vilnius, Lithuania.

2. **Vamze J**., Pilmane M., Skagers A. Analysis of cellular death in the experimental bone tissue regarding the biomaterial implantation [oral presentation] // Annals of Anatomy, 2014; 196 (S1): 88–89; 18th Congress of International Federation of Associations of Anatomists/30th Congress of Chinese Society of Anatomical Science, August 6–8, 2014, Beijing, China.
3. **Vamze J**., Pilmane M., Skagers A. Bone tissue morphological characteristic by using rutine investigation methods and Exact grunding system after the implantation of various bioceramic materials [poster presentation] // Abstract book: p. 53; Baltic Morphology VII Scientific Conference November 07–09, 2013, Riga, Latvia.
4. **Vamze J**., Pilmane M., Skagers A. Bone regeneration and host defense in rabbit bone after the implantation of pure and mixed hydroxyapatite and tricalcium phosphate [poster presentation] // Virchows Archiv, 2013; p. 323; Congress of European Society of Pathology, August 31–September 4, 2013, Lisbon, Portugal.
5. **Vamze J**., Pilmane M., Skagers A. Expression of bone regeneration proteins in rabbit bone tissue after the implantation of various bioceramic materials [poster presentation] // Abstract book: p. 29.; International Symposium on Bioceramics and Cells for Reinforcement of Bone, October 18–20, 2012, Riga, Latvia.
6. **Vamze J**., Pilmane M., Skagers A. Activity of host defense proteins in rabbit bone after pure hydroxyapatite and tricalcium phosphate and mixed tricalcium phosphate/hydroxyapatite implantation [poster presentation] // Scientific programm book: p. 35; International Symposium on Biomedical Engineering and Medical Physics, October 10–12, 2012, Riga, Latvia.

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1. **Vamze J.**, Pilmane M., Skaģers A. Apoptozes raksturojums truša kaulaudos pēc dažādu biomateriālu implantācijas [poster presentation] // Tēžu grāmata: 325. lpp.; Rīgas Stradiņa universitātes zinātniskā konference, 10.–11.04. 2014., Rīga, Latvija.
2. **Vamze J.**, Pilmane M., Skaģers A. Audu reaktogenitātes analīze trušu apakšžokļa kaulā pēc "tīra" un jaukta hidroksiapatīta un α-trikalcija fosfāta implantācijas [poster presentation] // tēzes elektroniski [www.arstubiedriba.lv]; Latvijas ārstu 7. kongress, 19.–20.09.2013., Rīga, Latvija.
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4. **Vamze J**., Pilmane M., Skaģers A., Šalms Ģ., Irbe Z. Kaula morfoģenētiskā proteīna, osteoprotegerīna, osteopontīna, osteokalcīna ekspresija truša apakšžokļa un stilba kaulaudos pēc dažādu biokeramikas materiālu implantācijas [oral presentation] // Tēžu grāmata: 299. lpp.; Rīgas Stradiņa universitātes 11. zinātniskā konference, 29.–30.03.2012.
5. **Vamze J**., Pilmane M. Kaulaudu reģeneratīvo procesu noteicošo faktoru izmaiņas truša apakšžokļa kaulā pēc HAp (hidroksiapatīta) implanta [poster presentation] // Tēžu grāmata: 330. lpp.; Rīgas Stradiņa universitātes 10. zinātniskā konference, 14.–15.04.2011., Rīga, Latvija.

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