Jānis Zariņš

ORCID 0000-0003-1168-0921

Changes in Bone Structure Following Implantation of Biphasic and Triphasic Strontium Enriched Biomaterials in Animals with Experimental Osteoporosis

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The Doctoral Thesis was developed at the Department of Morphology, Institute of Anatomy and Anthropology, Rīga Stradiņš University, Latvia

Supervisor of the Doctoral Thesis:

Dr. med., Dr. habil. med., Professor **Māra Pilmane**, Rīga Stradiņš University, Latvia

Dr. med. **Elga Sidhoma**, Rīga Stradiņš University, Latvia

Scientific Advisor:

Dr. med. **Ilze Šalma**, Rīga Stradiņš University, Latvia

Official Reviewers:

Dr. sc. ing., Associate Professor **Kristīne Šalma-Ancāne**, Rudolfs Cimdins Riga Biomaterials Innovations and Development Centre of Riga Technical University, Latvia

Dr. med., Professor **Ingrida Balnytė**, Lithuanian University of Health Sciences

Dr. med., Professor **Andres Arend**, University of Tartu, Estonia

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The Doctoral Thesis is available in RSU Library and on RSU website: https://www.rsu.lv/en/dissertations

Secretary of the Promotion Council:

Dr. habil. sc. ing., Profesor **Vladimir Kasjanov**

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List of abbreviations

BMP-2/4 – bone morphogenetic protein-2/4

Ca – calcium

CPC – calcium phosphate ceramics

Col-1α – collagen 1 alpha

ECM – extracellular matrix

HA – hydroxyapatite

Il-1 – interleukin-1

Il-6 – interleukin-6

Il-10 – interleukin-10

MMPs – matrix metalloproteinases

MMP-2 – matrix metalloproteinase-2

NFkB-105 – nuclear factor kappa beta-105

OC – osteocalcin

OPG – osteoprotegerin

RANK – receptor activator of nuclear factor kappa beta

RANKL – receptor activator of nuclear factor kappa beta ligand

Sham – empty control group, surgery without biomaterial implantation

Sr – strontium

TCP – tricalcium phosphate

TIMP-2 – tissue inhibitor of metalloproteinase-2

Introduction

Bone physical and morphological properties are provided by interaction between osteoblasts and osteoclasts. It is regulated by many biologically active factors, expression of which could be influenced by different biomaterials (Rucci, 2008). Calcium phosphate ceramics (*CPC*), composed of *HA* and *TCP*, have been widely used as bone substitutive materials due to the chemical properties similar to human bone (Praksam et al., 2017). Biphasic *CPC* biomaterials are able to exchange ions and bind them to provide bone homeostasis (Cardemil et al., 2013). Thereby, replacement of calcium (*Ca)* by other metal ions can locally advance bone regeneration (Offermanns et al., 2018). One of them is strontium (*Sr*) which has shown biological properties similar to *Ca*; therefore, it can affect bone metabolism (Andersen et al., 2013). *Sr* can simultaneously increase osteoblastogenesis and new bone formation, while decreasing osteoclastogenesis and bone resorption (Billström et al., 2014). Unique properties of *Sr* on bone tissue has led to its application in the development of new biomaterials. However, it is important to understand bone structural changes and interaction of biologically active factors during the bone regeneration.

One of the most important factors is osteocalcin (*OC*), which is expressed by osteoblasts; it regulates mineralisation of bone matrix (Rodrigues et al., 2012). Collagen 1 alpha (*Col-1α*) is the most abundant protein of extracellular matrix (*ECM*), which is synthesised by osteoblasts and forms a scaffold for apatite crystals (Ferreira et al., 2012). Osteoprotegerin (*OPG*) regulates osteoclastogenesis via inhibition of osteoclast precursor differentiation and bone resorption by preventing *RANKL* (receptor activator of nuclear factor kappa B ligand) from binding to its receptor *RANK* (receptor activator of nuclear factor kappa B) (Walsh and Choi, 2014). Whereas, *NFkB-105* regulates expression of different cytokine and during bone remodelling, it activates osteoclastogenesis

via *RANKL*/*RANK* mechanisms (Baud'huin et al., 2007). Bone morphogenetic protein-2/4 (*BMP-2/4*) is a multifunctional growth factor which regulates differentiation of mesenchymal stem cells into osteoblasts and improves new bone formation (Kim et al., 2015). Interaction of matrix metalloproteinase-2 (*MMP-2*), which is zinc dependent proteolytic enzyme, and tissue inhibitor of metalloproteinase-2 (*TIMP-2*) regulates turnover of bone matrix components, especially degradation of collagen (Baker et al., 2002; Davis and Seger, 2005). Bone regeneration is also controlled by local bone immunity. Interleukin-1 (*Il-1*) as a pro-inflammatory cytokine directly stimulates osteoclast differentiation via increasing expression of *RANKL* (Compston, 2001). Whereas, interleukin-10 (Il-10) as anti-inflammatory cytokine inhibits expression of *Il-1* and interleukin-6 (*Il-6*). During the bone remodelling, *IL-10* prevents bone resorption by suppressing *RANK/RANKL* induced osteoclastogenesis (Liu, Yao and Wise, 2006).

Although previous studies have demonstrated *Sr* substituted biomaterial effects on bone regeneration, there is lack of studies on osteoporotic rabbits, where level of the experimental osteoporosis is analogue to postmenopausal bone disease. Consequently, there is need for further investigations and comprehensions on *Sr* enriched *CPC* biomaterial induced bone structural changes in healthy and osteoporotic rabbits' bone.

Aim of the study and objectives

Therefore, the aim of the study was to evaluate and compare bone remodelling, mineralisation, growth factor, cytokine and osteoclastogenesis related factor distribution in healthy and osteoporotic rabbits' bone after implantation of biphasic or triphasic *Sr* ion enriched biomaterial implantation.

To achieve the aim, following objectives were proposed:

1) evaluation of morphological bone changes in healthy and osteoporotic bone;
2) analysis of immunohistochemical distribution of following factors related to bone homeostasis in healthy and osteoporotic bone:
    - osteocalcin (*OC*) – bone mineralisation factor;
    - collagen 1 alpha (*Col-1α)* – most abundant protein of bone matrix;
    - osteoprotegerin (*OPG*) – inhibitor of osteoclastogenesis;
    - nuclear factor kappa beta-105 (*NFkB-105*) – osteoclast activator, cellular activity indicator;
    - bone morphogenetic protein-2/4 (*BMP-2/4*) – bone growth and regeneration related factor;
    - matrix metalloproteinases-2 (*MMP-2*) – bone matrix degrading enzyme;
    - tissue inhibitor of matrix metalloproteinase-2 (*TIMP-2*) – inhibitor of bone matrix degrading enzyme;
    - interleukin-1 (*Il-1*) – inflammatory cytokine and bone resorption related factor;
    - interleukin-10 (*Il-10*) – anti-inflammatory cytokine and bone local immunity related factor;
3) comparison of results and estimate of statistical significance between study groups.

Hypothesis of the study

Strontium enriched biomaterials can better stimulate bone regenerative properties, improving bone homeostasis in osteoporotic bone conditions.

Novelty of the study

In this study for the first time 11 different bone tissue conditions were studied following single methodology. Healthy bone samples were compared with osteoporotic ones after *Sr-HA_{30}/TCP_{70}*, *HA_{30}/TCP_{70}*, *Sr-HA_{70}/TCP_{30}*, *HA_{70}/TCP_{30}* biomaterial implantation or *sham* surgery, as well as bone samples from non-operated leg. Moreover, many bone metabolism related factors as *OPG, OC, NFkB-105, BMP-2/4, Col-1α, MMP-2, TIMP-2, Il-1* un *Il-10* were evaluated. Number of analysed bone samples and variety of factors are more extensive compared to published studies in literature and some of them have been studied for the first time. Obtained data could promote further investigation of *Sr* enriched biomaterials in future studies, as well as, some of the analysed markers could be used in clinical practice for osteoporosis diagnostic, therapeutic and prognostic purposes.

Personal contribution

The author of this study has performed the immunohistochemical analysis, acquired scientific data and carried out statistical analysis. The author has written all of this scientific work and is the author of all microphotographs included in the study. More than 950 bone samples were analysed.

Ethical aspects

The experimental animal project was approved with the permission of Animal Ethics Committee of Food and Veterinary Administration of Latvia (No 72). It was issued for a National research programme "Multifunctional materials and composites, photonics and nanotechnologies (*IMIS2*) project "Nanomaterials and nanotechnologies for medical purposes"".

Structure and volume of the Doctoral Thesis

The Doctoral Thesis was written in Latvian. It consists of five chapters: review of literature, material and methods, results, discussion and conclusions. The list of references consists of 222 sources. The volume of the Doctoral Thesis covers 168 pages, including 35 tables and 107 figures (microphotographs).

1 Materials and methods

1.1 Study groups

Forty-six matured rabbits (species: California and Big Marder; sex: female; age: 8 months) were used for the study.

Rabbits were divided into four experimental groups (See Table 1.1):

1) in group **A**, all rabbits were healthy, not operated and formed the control group. In group **B**, **C** and **D** all rabbits underwent induction of osteoporosis;
2) in group **B** and **C**, bone defect of right femur was filled with and without 5 % *Sr* enriched HA_{30}/TCP_{70} and HA_{70}/TCP_{30} granules;
3) in group **D**, bone defect was left empty and formed *sham* group.

Table 1.1

Study design

Group	**A**	**B**		**C**		**D**
Status	Healthy	*Sr-HA/TCP*	*HA/TCP*	*Sr-HA/TCP*	*HA/TCP*	*Sham*
Phase ratio	-	30/70	30/70	70/30	70/30	-
Number of animals	10	7	7	7	8	7

Abbreviations: *Sr* – strontium, *HA* – hydroxyapatite, *TCP* – tricalcium phosphate.

1.2 Induction of experimental osteoporosis

Protocol and dosage of medication is based on previously described principles of methodology (Baofeng et al., 2010; Meredith, 2015). The midline laparotomy and ovarectomy was performed under general anesthesia (ketamine 10 % 30 mg/kg, xylazine 2 % 3 mg/kg and atropine 0.1 % 0.1–0.5 mg/kg.

Postoperative pain management was controlled by subcutaneous ketoprofen (1–3 mg/kg) injections once a day. Assessment of the rabbits was performed two weeks later. When wounds were healed, intramuscular injections of methylprednisolone at a dose of 1 mg/kg were done daily for six weeks. During the experiment all rabbits were allowed for voluntary movements and nutrition.

1.3 Characterisation of biomaterials

Non-commercial biomaterials obtained from Rudolfs Cimdins Riga Biomaterials Innovations and Development Centre of Riga Technical University were used. The Sr substituted and non-substituted biphasic *CPC* granules were prepared and phase composition analysed according to the procedures described in literature (Stipniece et al., 2016; Grybauskas et al., 2015). Calcium deficit hydroxyapatite precipitates were harvested via variation of synthesis parameters such as temperature, pH of synthesis and concentration of reagents. X-ray diffraction analysis (*PANalytical X'Pert PRO*) did not show any foreign phases in all samples. The field emission scanning electron microscopy analysis (*FE-SEM, Mira/LMU, Tescan*) demonstrated sintered granules in irregular and different shapes, in size range from 0.5 to 1 mm. Micro porosity and grain size of granules was in the range from 400 nm to 1 μm. Significant macrostructural and microstructural differences of analysed granules were not detected.

1.4 Implantation of biomaterials

Under general anesthesia (protocol described in section 1.2) soft tissue and periosteum from right trochanter major of the femur bone were divided and 5 mm wide bone defect was made using trephine burr drill. Bone defect was filled with biomaterial granules or left empty as described in Table 1.1.

1.5 Morphological investigation of the materials

Euthanasia was performed 12 weeks after the second surgery. Bone samples were taken from operated leg and from non-operated leg to assess local and generalised tissue response to biomaterials and the surgery. No visual changes of hip joint cartilage were observed at the time of sacrifice. Bone samples were obtained from identical anatomical location of the healthy rabbits.

Bone samples were fixed in *Stefanini's* solution and kept in refrigerator (4 °C) for at least 24 hours. Bone samples were decalcified with *Decalcifier Rapid* (code ITB RS 155800054, *I.T. Baker*, Netherlands) solution. Dehydration was performed using alcohol solutions and deprived using xylol solution. Bone samples were embedded in paraffin cassettes, then 3–5 μm tissue sections were prepared using microtome (*Leica, RM2245 Leica Biosystems Richmond Inc.*, USA) and put on slides for further processing. *Diapath* slides (*Diapath*, Italy) were used for hematoxylin and eosin staining, but *Histobond* slides (*Marienfield*, Germany) for immunohistochemical method.

1.5.1 Routine histological staining method

Bone samples were processed for routine histological staining to assess morphological overview of them (Fischer, 2008). Before staining, bone sections were deparaffinised in ortoxylol solution, then dehydrated in 70° and 96° alcohol solution and then rinsed in distilled water. Afterwards, staining in hematoxylin solution (*Mayers Hematoxylin*, code 05M06002, *Bio-Optica*, Italy) and in eosin solution (*Eosin Y Alcoholic solution*, code 05B1003, *Bio-Optica*, Italy) were performed. Then following rinsing in distilled water, dehydration in 70° and 96° alcohol solution, clarifying with carboxylic acid and ortoxylol solution was carried out. Finally, sections were covered with polystyrole for coverslip

attachment. According to this method, nucleus of the cells stained as basophilic in blue – violet color, but cytoplasm stained as acidophilic in pink.

1.5.2 Immunohistochemical method and reagents

Bone tissue sample preparation and placement onto slides was done according to protocol mentioned in section 1.5. Immunohistochemical analysis was carried out using biotin-streptavidin method (Hsu, Raine and Fanger, 1981). At the beginning deparaffinisation was done in xylol solution, then rinsing in alcohol and distilled water. Washing of samples was performed using TRIS buffer solution (*TWEEN20*, code T0083, *Diapath*, Italy) and boiling – using EDTA buffer solution (code TU103, *Diapath*, Italy). Further, samples were cooled down and processed with 3 % peroxidase solution for 10 minutes. Afterwards, they were rinsed in distilled water and TRIS buffer solution. Background staining was diminished using normal blocking serum for 20 minutes (code 9273-05, *Cell Marque*, United States of America). The following step was incubation of all samples with primary antibodies for 60 minutes in high humidity conditions (used antibodies are mentioned in Table 1.2). Subsequently, tissue samples were rinsed in TRIS buffer solution for 10 minutes, and then for 30 minutes; samples were incubated using LSAB + LINK biotin conjugated secondary antibody (code K1015, *DakoCytomation*, Denmark). Afterwards, samples were rinsed again in TRIS buffer solution and underwent processing for 25 minutes using LSAB + KIT with peroxidase enzyme conjugated streptavidin (code K0690, *DakoCytomation*, Denmark). Then reiterative rinsing with TRIS buffer solution and processing with DAB chromogenic substrate kit (code K3468, *DakoCytomation*, Denmark) were performed, resulting that all positive structures were stained in brown. Finally, all samples were rinsed in running

water and stained with hematoxylin for 2 minutes (*Mayers Hematoxylin*, code 05M06002, *BioOptica*, Italy).

Table 1.2

Used antibodies for immunohistochemical analysis

Factor	Source	Code	Working dilution	Producer, state
OPG	Rabbit	Orb120312	1:100	*Biorbyt, USA*
NFkB-105	Rabbit	AB7971	1:100	*Abcam, UK*
OC	Rabbit	Orb259644	1:100	*Biorbyt, USA*
Col-1α	Rabbit	Orb106535	1:100	*Biorbyt, USA*
BMP-2/4	Goat	AF355	1:100	*R&D systems, UK*
MMP-2	Rabbit	AF902	1:100	*Biorbyt, USA*
TIMP-2	Mouse	Sc-21735	1:100	*Santa Cruz, USA*
Il-1	Rabbit	Orb308737	1:100	*Biorbyt, USA*
Il-10	Rabbit	P22301	1:100	*ABBIOTEC, LLC, USA*

Abbreviations: *OPG* – osteoprotegerin, *NFkB-105* – nuclear factor kappa beta-105, *OC* – osteocalcin, *Col-1α* – collagen 1 alpha, *BMP-2/4* – bone morphogenetic protein-2/4, *MMP-2* – matrix metalloproteinase-2, *TIMP-2* – tissue inhibitor of matrix metalloproteinases-2, *Il-1* – interleukin-1, *Il-10* – interleukin-10, *USA* – United States of America, *UK* – United Kingdom.

1.6 Data processing methods

Bone sections were visualised using light microscopy (Leica DM500RB, Leica Biosystems Richmond Inc., USA) and photography was done by microscope with camera (Leica DC300F, Leica Microsystem AG, Germany).

1.6.1 Evaluation of bone area

Trabecular and intertrabecular bone area was measured using *ImagePro Plus-7* programme, where three equal fields (0.975 mm^2) of view were chosen. Osteoporosis index was defined as ratio between them.

1.6.2 Semiquantitative counting method

Semiquantitative counting was used to evaluate relative amount of immunopositive osteocytes (See Table 1.3) (Pilmane, Luts and Sandlers, 1995).

Table 1.3

Designation of semiquantitative counting method

Grading	Explanation	Usage for statistics
0	No positive structures in the visual field	0
0/+	Occasionally positive structures in the visual field	0.5
+	Few positive structures in the visual field	1
+/++	Few to moderate positive structures in the visual field	1.5
++	Moderate positive structures in the visual field	2
++/+++	Moderate to numerous positive structures in the visual field	2.5
+++	Numerous positive structures in the visual field	3
+++/++++	Numerous to abundant positive structures in the visual field	3.5
++++	Abundant positive structures in the visual field	4

1.6.3 Statistical methods

Statistical analysis was done using *Statistical Package for Social Sciences IBM* (SPSS) version 23 (*IBM Corporations*, USA). The Mann–Whitney U test and Spearman's rank correlation coefficient (r_s) were used. Correlation effect size was set as following: 0.00–0.19 "very week"; 0.20–0.39 "weak"; 0.40–0.59 "moderate"; 0.60–0.79 "strong" and 0.80–1.0 "very strong". Evaluations were two-tailed and value of $p < 0.05$ was considered statistically significant.

2 Results

Death of two rabbits was detected during the research. Both animals died after the second surgery. One of them died after the implantation of HA_{70}/TCP_{30}, but another after the *sham* surgery. The damage of visceral organs was not found during the pathohistological examination. Association between the operation and death of both animals was not proved. Therefore, tissue samples from 44 animals were analysed.

2.1 Morphological findings

Normal histological structure, consisting of compact bone with osteon channels, blood vessels inside and spongy bone with prominent bony trabeculae, osteocytes and red bone marrow with adipocytes was obtained in all tissue sections of control or group A (see Fig. 2.1 A).

Different bone structure was detected in osteoporotic rabbits. The compact bone layer was thinner and contained less osteons, with channels more pronounced proliferation of connective tissue. Also the bony trabeculae were prominently thinner and sparser. Red bone marrow contained prominently more adipocytes. Such changes were observed in both operated and non-operated leg tissue samples. Implanted granules, localised newly formed bone, focal connective tissue, giant cells and osteoclasts were observed after pure and Sr enriched biomaterial implantation (see Fig. 2.1 B, C, D, E). Presence of osteoclasts was not observed in the tissue samples from *sham* group (see Fig. 2.2). In general, more pronounced presence of connective tissue and fewer adipocytes were obtained in the regeneration zone of the bone (see Fig. 2.1 F).

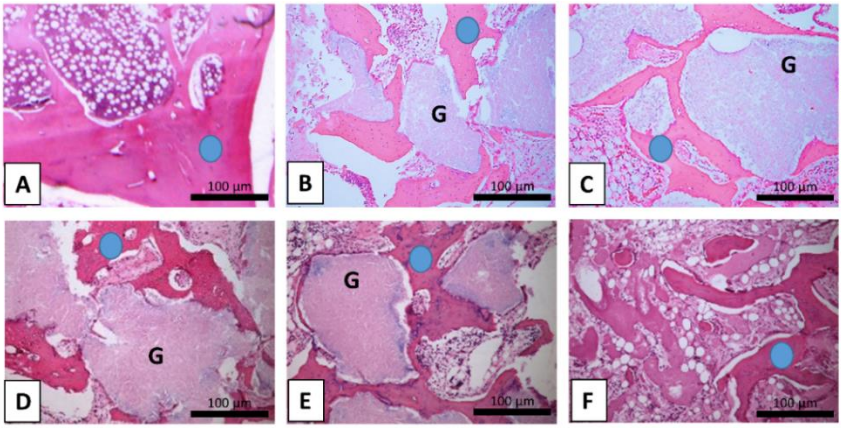

Fig. 2.1 **Visual comparison of healthy and operated rabbit bone structure***

*A – prominent bony trabeculae in the control group; B – *Sr-HA*$_{30}$*/TCP*$_{70}$ tissue sample; C – *HA*$_{30}$*/TCP*$_{70}$ tissue sample; D – *Sr-HA*$_{70}$*/TCP*$_{30}$ tissue sample; E – *HA*$_{70}$*/TCP*$_{30}$ tissue sample; F – thin and sparse bony trabeculae with many adipocytes in *sham* tissue sample. B, C, D and E – thin and sparse bony trabeculae, sparse osteons, prominent connective tissue. G – granules; blue dot – bony trabecula. *H&E*, × *100*, scale 100 µm.

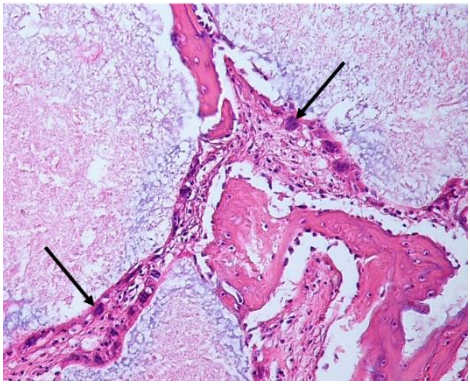

Fig. 2.2 **Tissue sample from rabbits *Sr-HA*$_{30}$*/TCP*$_{70}$ operated leg with osteoclasts (arrows) around implanted granules. *H&E*, × 100**

2.2 Bone area measurements

The bone trabecular area was 0.393 mm^2 in the rabbits of control group, which was significantly higher than in osteoporotic ones. **The bone intertrabecular area** was 0.582 mm^2 in the rabbits of control group, which was significantly lower than in osteoporotic rabbits (see Table 2.1). Statistically significant difference was not observed between the tissue samples after biomaterial implantation, sham or intact leg tissue.

The osteoporosis index was 68 % in the rabbits of control group, which was statistically significantly higher than in the tissue of operated leg in osteoporotic rabbits. The osteoporosis index was 32 % (p = 0.005) in *Sr-HA_{30}/TCP_{70}*, it was 30 % (p = 0.001) in *HA_{30}/TCP_{70}*, it was 27 % (p = 0.001) in *Sr-HA_{70}/TCP_{30}*, it was 30 % (p = 0.001) in *HA_{70}/TCP_{30}* and it was 33 % (p = 0.005) in *sham* tissue.

Table 2.1

The trabecular and intertrabecular area of osteoporotic rabbits

Status	B		C		D
	Sr-HA_{30}/TCP_{70}	***HA_{30}/TCP_{70}***	***Sr-HA_{70}/TCP_{30}***	***HA_{70}/TCP_{30}***	***Sham***
Operated leg	[a] 0.23 [b] 0.74	[e] 0.23 0.75	0.21 0.76	0.22 0.75	0.24 0.74
Intact leg	0.23 0.75	0.20 0.84	0.22 0.76	0.21 0.76	0.21 0.79
	[c] p = 0.001 [d] p = 0.001	p = 0.001 p = 0.001	p = 0.001 p = 0.001	p < 0.001 p = 0.001	p = 0.005 p < 0.001

Abbreviations: *Sr* – strontium, *HA* – hydroxyapatite, *TCP* – tricalcium phosphate.
[a]the bone trabecular area; [b]the bone intertrabecular area; [c]statistically significant difference between bone trabecular area in legs of healthy and osteoporotic rabbits; [d]statistically significant difference between bone intertrabecular area in legs of healthy and osteoporotic rabbits, [e]measurement in mm^2.

2.3 Immunohistochemical profile of analysed factors in control and osteoporotic animal bone

2.3.1 Bone matrix and mineralisation factors

On average, few to moderate number (+/++) of *Col-1α* positive osteocytes were obtained in the control or **group A** (see Fig. 2.3).

On average, numerous (+++) *Col-1α* positive bone cells were obtained in the tissue samples of **group B** *Sr-HA$_{30}$/TCP$_{70}$* operated leg. Non-operated leg overall contained moderate number (++) of *Col-1α* positive structures. **Statistically significantly more** *Col-1α* positive structures were obtained in the tissue of operated leg than in non-operated leg (U = 7.50; p = 0.015). **Statistically significantly more** *Col-1α* positive structures were seen in the operated leg compared to the control group (U = 0.50; p = 0.001).

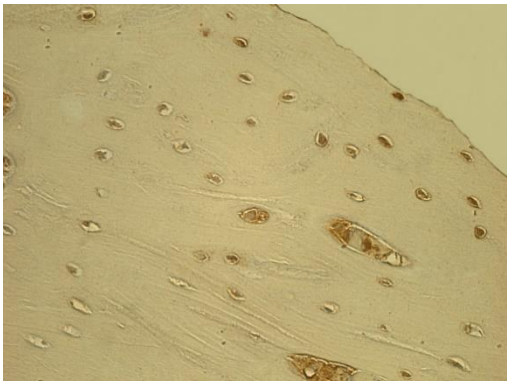

Fig. 2.3 **Moderate number of *Col-1α* positive osteocytes in the femur bone of the control group rabbit. *Col-1α IMH*, × 400**

On average, numerous (+++) *Col-1α* positive structures were obtained in the tissue of **group B** *HA$_{30}$/TCP$_{70}$* operated leg. Non-operated leg overall contained moderate to numerous (++/+++) *Col-1α* positive structures.

Statistically significant difference was not found between the number of *Col-1α* positive structures of operated and non-operated leg (U = 18.50; p = 0.405). **Statistically significantly more** *Col-1α* positive structures were obtained in the operated leg compared to the control group (U = 0.50; p = 0.001).

On average, numerous (+++) *Col-1α* positive bone cells were obtained in the tissue samples of **group C** *Sr-HA$_{70}$/TCP$_{30}$* operated leg (see Fig. 2.4). Non-operated leg on average contained few to moderate number (+/++) of *Col-1α* positive cells. **Statistically significantly more** *Col-1α* positive structures were found in the operated leg than in non-operated leg (U = 4.00; p = 0.02). **Statistically significantly more** *Col-1α* positive structures were obtained in the operated leg compared to the control group (U = 0.50; p = 0.001).

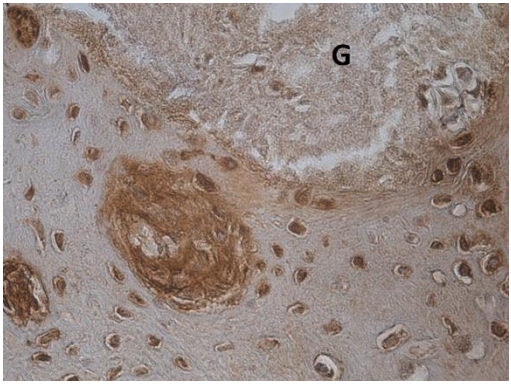

Fig. 2.4 **Numerous *Col-1α* positive osteocytes in the operated leg bone of *Sr-HA$_{70}$/TCP$_{30}$* rabbit. G – granule. *Col-1α IMH*, × 400**

On average, moderate to numerous (++/+++) *Col-1α* positive structures were obtained in the tissue of **group C** *HA$_{70}$/TCP$_{30}$* operated leg. Non-operated leg contained moderate number (++) of *Col-1α* positive cells. **Statistically**

significantly more *Col-1α* positive structures were seen in the operated leg than in the non-operated leg U = 5.00; p = 0.03). **Statistically significantly more** *Col-1α* positive structures were obtained in the operated leg compared to the control group (U = 0.50; p < 0.001).

On average, numerous (+++) *Col-1α* positive structures were obtained in the tissue samples of **group D** *sham* operated leg. Non-operated leg contained moderate number (++) of *Col-1α* positive cells. **Statistically significantly more** *Col-1α* positive structures were seen in the operated leg than in the non-operated leg (U = 6.00; p = 0.044). **Statistically significantly more** *Col-1α* positive structures were obtained in the operated leg compared to the control group (U = 0.50; p = 0.001).

The number of ***Col-1α*** positive cells **was not significantly different** between operated osteoporotic rabbits from **groups B, C and D.**

On average, numerous (+++) ***OC*** positive osteocytes were obtained in the control or **group A**.

On average, numerous (+++) *OC* positive bone cells were obtained in the tissue samples of **group B** *Sr-HA_{30}/TCP_{70}* operated leg. Non-operated leg overall contained moderate number (++) of *OC* positive cells. **Statistically significantly more** *OC* positive structures were found in the operated leg than in the non-operated leg (U = 4.50; p = 0.005). **Statistically significant difference was not found** between the number of *OC* positive structures in the control group and operated leg (U = 25.50; p = 0.212).

On average, moderate to numerous (++/+++) *OC* positive structures were obtained in the tissue of **group B** *HA_{30}/TCP_{70}* operated leg. Moderate number (++) of *OC* positive cells was observed in the tissue of non-operated leg. **Statistically significant difference was not found** between the number of *OC* positive structures in the operated and non-operated leg (U = 12.00; p = 0.097). **Statistically significant difference was not found** between the

number of *OC* positive structures in the control group and operated leg (U = 20.50; p = 0.079).

The highest expression of *OC* was obtained in the tissue of **group C** *Sr-HA$_{70}$/TCP$_{30}$* operated leg. On average numerous to abundant (+++/++++) *OC* positive bone cells were found (see Fig. 2.5). Non-operated leg overall contained few to moderate number (+/++) of *OC* positive cells (see Fig. 2.6). **Statistically significantly more** *OC* positive structures were obtained in the operated leg than in the non-operated leg (U = 1.00; p = 0.006). **Statistically significantly more** *OC* positive structures were found in the operated leg compared to the control group (U = 10.50; p = 0.023).

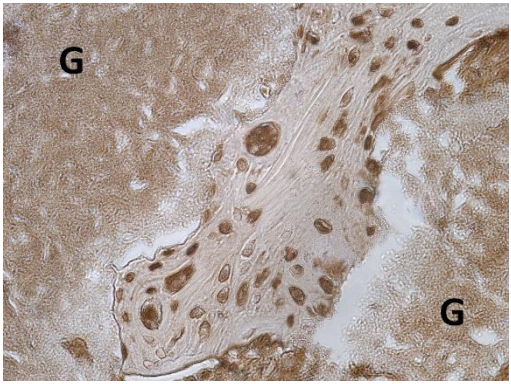

Fig. 2.5 **Numerous to abundant *OC* positive osteocytes in the operated leg bone of *Sr-HA$_{70}$/TCP$_{30}$* rabbit. G – granule. *OC IMH*, × 250**

On average, numerous (+++) *OC* positive structures were found in the tissue of **group C** *HA$_{70}$/TCP$_{30}$* operated leg. Non-operated leg overall contained moderate number (++) of *OC* positive cells. **Statistically significantly more** *OC* positive structures were found in the operated leg than in the non-operated leg (U = 2.50; p = 0.001). **Statistically significant difference was not found**

between the number of *OC* positive structures in the control group and operated leg (U = 35.50; p = 0.613).

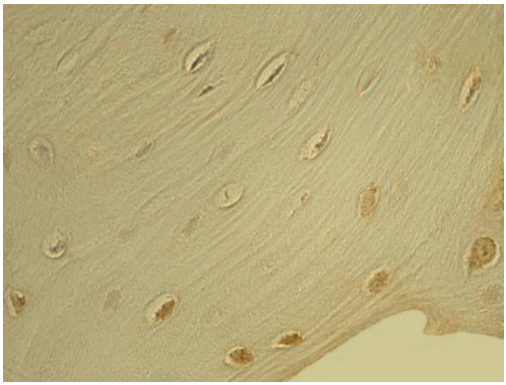

Fig. 2.6 **Few to moderate number of *OC* positive osteocytes in the non-operated leg bone of *Sr-HA$_{70}$/TCP$_{30}$* rabbit. *OC IMH*, × 400**

On average, moderate to numerous (++/+++) *OC* positive structures were found in the tissue samples of **group D** *sham* operated leg. Non-operated leg overall contained moderate number (++) of *OC* positive cells. **Statistically significant difference was not found** between the number of *OC* positive structures in the operated and non-operated leg (U = 9.00; p = 0.105). Meanwhile, the tissue samples of operated leg contained **statistically significantly less** *OC* positive structures compared to the control group (U = 15.00; p = 0.047).

Statistically significantly more *OC* positive bone cells were found after the implantation of ***Sr-HA$_{70}$/TCP$_{30}$*** granules compared to *Sr-HA$_{30}$/TCP$_{70}$* (U = 5.5; p = 0.022), *HA$_{30}$/TCP$_{70}$* (U = 4.5; p = 0.016) granules or to the *sham* surgery (U = 3.0; p = 0.014).

2.3.2 Bone regeneration and cellular activity factors

On average, few to moderate number (+/++) of ***BMP-2/4*** positive cells were found in the tissue of control or **group A** (see Fig. 2.7).

Moderate to numerous (++/+++) *BMP-2/4* positive bone cells were obtained in the tissue samples of **group B** *Sr-HA_{30}/TCP_{70}* operated leg. Non-operated leg on average contained few to moderate number (+/++) of *BMP-2/4* positive cells. **Statistically significantly more** *BMP-2/4* positive structures were found in the operated leg than in the non-operated leg ($U = 7.00$; $p = 0.021$). **Statistically significantly more** *BMP-2/4* positive structures were obtained in the operated leg compared to the control group ($U = 6.50$; $p = 0.004$).

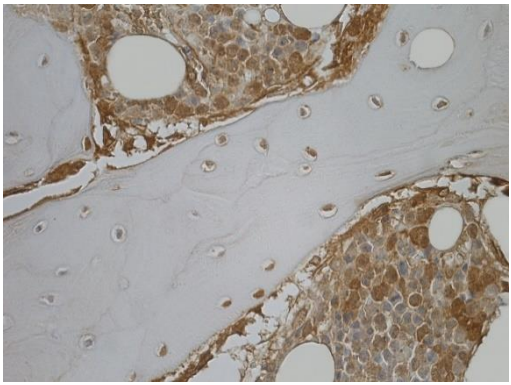

Fig. 2.7 **Few to moderate number of *BMP-2/4* positive osteocytes in the femur bone of a control group rabbit. *BMP-2/4 IMH*, × 250**

On average, moderate number (++) of *BMP-2/4* positive structures was found in the tissue of **group B** *HA_{30}/TCP_{70}* operated leg. Similarly, moderate number (++) of positive cells was obtained in the non-operated leg. **Statistically significant difference was not found** between the number of *BMP-2/4* positive

structures in the operated and non-operated leg (U = 18.00; p = 0.295). **Statistically significantly more** *BMP-2/4* positive structures were found in the operated leg compared to the control group (U = 14.50; p = 0.031).

On average moderate to numerous (++/+++) *BMP-2/4* positive bone cells were found in the tissue samples of **group C** *Sr-HA$_{70}$/TCP$_{30}$* operated leg (see Fig. 2.8). Meanwhile, occasional (0/+) *BMP-2/4* positive cells were found in the non-operated leg. **Statistically significantly more** *BMP-2/4* positive structures were obtained in the operated leg than in the tissue of non-operated leg (U = 3.50; p = 0.003). **Statistically significantly more** *BMP-2/4* positive structures were obtained in the operated leg compared to the control group (U = 3.00; p = 0.002).

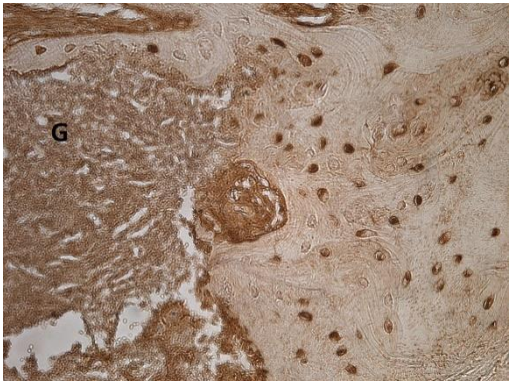

Fig. 2.8 **Moderate to numerous *BMP-2/4* positive osteocytes in the operated leg of *Sr-HA$_{70}$/TCP$_{30}$* rabbit. G – granule. *BMP-2/4 IMH*, × 250**

On average, moderate number (++) of *BMP-2/4* positive cells was found in the tissue of **group C** *HA$_{70}$/TCP$_{30}$* operated leg. Meanwhile, on average, occasional (0/+) *BMP-2/4* positive cells were obtained in the non-operated leg. **Statistically significantly more** *BMP-2/4* positive structures were found in the operated leg than in the non-operated leg (U = 1.00; p = 0.011). **Statistically**

significantly more *BMP-2/4* positive structures were obtained in the operated leg compared to the control group (U = 12.00; p = 0.009).

On average, moderate number (++) of *BMP-2/4* positive structures was found in the tissue of **group D** *sham* operated leg. Few to moderate number (+/++) of *BMP-2/4* positive cells were found in the non-operated leg. **Statistically significant difference was not found** between the number of *BMP-2/4* positive structures in the operated and non-operated leg (U = 8.50; p = 0.088). **Statistically significant difference was not found** between the number of *BMP-2/4* positive structures of the control group and operated leg (U = 13.50; p = 0.056).

Statistically significantly more *BMP-2/4* positive bone cells were found after the implantation of ***Sr-HA$_{70}$/TCP$_{30}$*** granules compared to the *HA$_{30}$/TCP$_{70}$* granules (U = 9.00; p = 0.035) or to the *sham* surgery (U = 7.50; p = 0.043).

On average, moderate number (++) of ***NFkB-105*** positive cells was found in the tissue of control or **group A**.

On average. moderate number (++) of *NFkB-105* positive bone cells was obtained in the tissue samples of **group B** *Sr-HA$_{30}$/TCP$_{70}$* operated leg. Non-operated leg, on average, contained few (+) *NFkB-105* positive cells. **Statistically significantly more** *NFkB-105* positive structures were found in the operated leg than in the non-operated leg (U = 0.50; p = 0.001). **Statistically significant difference was not found** between the number of *NFkB-105* positive structures in the control group and operated leg (U = 34.00; p = 0.895).

On average, few to moderate number (+/++) of *NFkB-105* positive structures were found in the tissue of **group B** *HA$_{30}$/TCP$_{70}$* operated leg (see Fig. 2.9). Meanwhile, on average, few (+) *NFkB-105* positive structures were obtained in the non-operated leg (see Fig. 2.10). **Statistically significantly more**

NFkB-105 positive structures were found in the operated leg than in the non-operated leg (U = 9.00; p = 0.03). Meanwhile, the tissue samples of operated leg contained **statistically significantly less** *NFkB-105* positive structures compared to the control group (U = 9.50; p = 0.007).

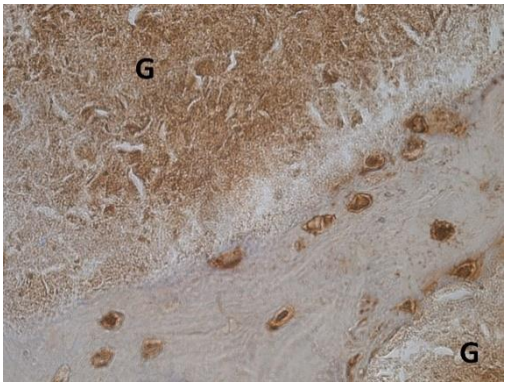

Fig. 2.9 **Few to moderate number of *NFkB-105* positive osteocytes in the operated leg of HA_{30}/TCP_{70} rabbit. G – granule. *NFkB-105 IMH*, × 400**

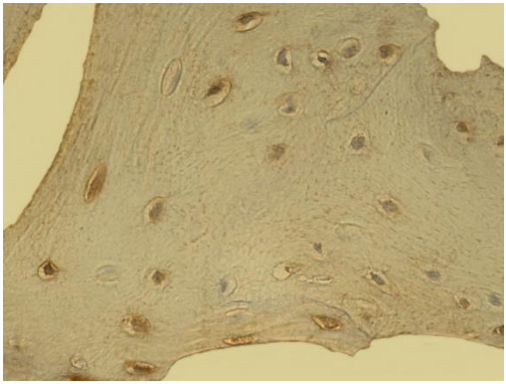

Fig. 2.10 **Few *NFkB-105* positive osteocytes in the non-operated leg bone of HA_{30}/TCP_{70} rabbit. *NFkB-105 IMH*, × 400**

More pronounced expression of *NFkB-105* was obtained in the tissue samples of **group C** ***Sr-HA$_{70}$/TCP$_{30}$*** operated leg. On average, numerous (+++) *NFkB-105* positive bone cells were found. Non-operated leg overall contained moderate number (++) of *NFkB-105* positive cells. **Statistically significantly more** *NFkB-105* positive structures were found in the operated leg than in the non-operated leg (U = 3.00; p = 0.012). **Statistically significantly more** *NFkB-105* positive structures were obtained in the operated leg compared to the control group (U = 10.00; p = 0.016).

On average, moderate number (++) of *NFkB-105* positive structures was found in the tissue of **group C** *HA$_{70}$/TCP$_{30}$* operated leg. Overall, few to moderate number (+/++) of *NFkB-105* positive structures was observed in the non-operated leg. **Statistically significantly more** *NFkB-105* positive structures were obtained in the operated leg than in the non-operated leg (U = 10.00; p = 0.011). **Statistically significant difference was not found** between the number of *NFkB-105* positive structures in the control group and operated leg (U = 24.60; p = 0.10).

On average, moderate number (++) of *NFkB-105* positive structures was found in the tissue samples of **group D** *sham* operated leg. Moderate number (++) of *NFkB-105* positive bone cells was also found in the non-operated leg. **Statistically significant difference was not found** between the number of *NFkB-105* positive structures in the operated and non-operated leg (U = 16.50; p = 0.794). **Statistically significant difference was not found** between the number of *NFkB-105* positive structures in the control group and operated leg (U = 18.50; p = 0.148).

Statistically significantly more ***NFkB-105*** positive bone cells were observed after the implantation of ***Sr-HA$_{30}$/TCP$_{70}$*** granules compared to the *HA$_{30}$/TCP$_{70}$* granules (U = 6.0; p = 0.01).

Statistically significantly more ***NFkB-105*** positive bone cells were observed after the implantation of ***Sr-HA$_{70}$/TCP$_{30}$*** granules compared to *Sr-HA$_{30}$/TCP$_{70}$* (U = 6.0; p = 0.014) and *HA$_{30}$/TCP$_{70}$* (U = 1.0; p = 0.003) granules or to the *sham* operation (U = 3.0; p = 0.012).

Statistically significantly more ***NFkB-105*** positive bone cells were observed after the implantation of ***HA$_{70}$/TCP$_{30}$*** granules compared to the *HA$_{30}$/TCP$_{70}$* (U = 2.5; p = 0.002) or to the *sham* surgery (U = 7.5; p = 0.018).

Overall, moderate to numerous (++/+++) ***OPG*** positive cells were obtained in the tissue of control or **group A**.

Numerous (+++) *OPG* positive bone cells were obtained in the tissue samples of **group B** Sr-*HA$_{30}$/TCP$_{70}$* operated leg (see Fig. 2.11). Non-operated leg overall contained moderate number (++) of *OPG* positive cells. **Statistically significantly more** *OPG* positive structures were obtained in the operated leg than in the non-operated leg (U = 5.00; p = 0.007). **Statistically significant difference was not found** between the number of *OPG* positive structures in the control group and operated leg (U = 29.50; p = 0.561).

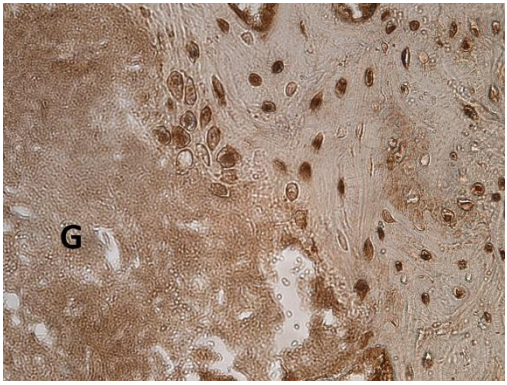

Fig. 2.11 **Numerous *OPG* positive osteocytes in the operated leg bone of *Sr-HA$_{30}$/TCP$_{70}$* rabbit. G – granule. *OPG IMH*, × 250**

On average, moderate number (++) of *OPG* positive structures was obtained in the tissue of **group B** HA_{30}/TCP_{70} operated leg (see Fig. 2.12). On average moderate number (++) of *OPG* positive cells was also observed in the non-operated leg. **Statistically significant difference was not found** between the number of *OPG* positive structures in the operated and non-operated leg (U = 21.00; p = 0.60). The tissue samples of operated leg contained **statistically significantly less** *OPG* positive structures compared to the control group (U = 14.50; p = 0.033).

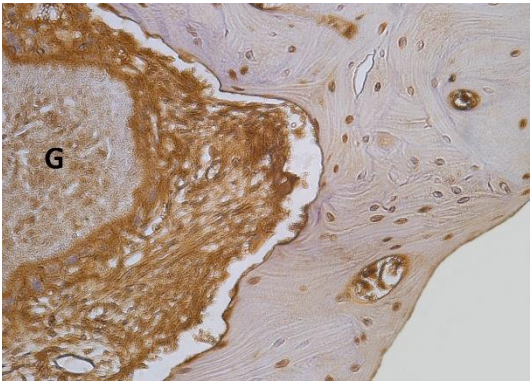

Fig. 2.12 **Moderate number of *OPG* positive osteocytes in the operated leg bone of HA_{30}/TCP_{70} rabbit. G – granule. *OPG IMH*, × 250**

On average, numerous (+++) *OPG* positive bone cells were obtained the tissue samples of **group C** *Sr-*HA_{70}/TCP_{30} operated leg. Non-operated leg overall contained moderate number (++) of *OPG* positive cells. **Statistically significantly more** *OPG* positive structures were obtained in the operated leg than in the non-operated leg (U = 3.00; p = 0.002). **Statistically significant difference was not found** between the number of *OPG* positive structures in the control group and operated leg (U = 20.00; p = 0.232).

On average, moderate number (++) of *OPG* positive cells was obtained in the tissue of **group C** HA_{70}/TCP_{30} operated leg. Non-operated leg contained moderate number (++) of *OPG* positive cells. **Statistically significantly more** *OPG* positive cells were obtained in the operated than in the non-operated leg (U = 15.00; p = 0.029). Operated leg contained **statistically significantly less** *OPG* positive structures compared to the control group (U = 19.00; p = 0.049).

Moderate number (++) of *OPG* positive cells was observed in the tissue samples of **group D** *sham* operated leg. Overall, moderate number (++) of *OPG* positive cells was also found in the tissue samples of non-operated leg. **Statistically significant difference was not found** between the number of *OPG* positive structures in the operated and non-operated leg (U = 15.0; p = 0.523). Operated leg contained **statistically significantly less** *OPG* positive structures compared to the control group (U = 10.0; p = 0.018).

Statistically significantly more *OPG* positive bone cells were observed after the implantation of ***Sr-HA₃₀/TCP₇₀*** granules compared to the HA_{30}/TCP_{70} (U = 9.5; p = 0.04) or HA_{70}/TCP_{30} (U = 13.0; p = 0.048) granules, or to the *sham* surgery (U = 6.0; p = 0.023).

Statistically significantly more *OPG* positive bone cells were observed after the implantation of ***Sr-HA₇₀/TCP₃₀*** compared to the HA_{30}/TCP_{70} (U = 5.0; p = 0.015) or HA_{70}/TCP_{30} (U = 8.0; p = 0.025) granules, or to the *sham* surgery (U = 2.0; p = 0.007).

2.3.3 Bone degrading enzymes and their inhibitors

Overall, moderate number (++) of ***MMP-2*** positive cells was obtained in the tissue of control or **group A**.

Overall, moderate number (++) of *MMP-2* positive bone cells was obtained in the tissue samples of **group B** *Sr-*HA_{30}/TCP_{70} operated leg (see Fig.

2.13). Non-operated leg on average contained few to moderate number (+/++) of *MMP-2* positive cells. **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the operated and non-operated leg (U = 15.50; p = 0.219). **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the control group and operated leg (U = 27.50; p = 0.450).

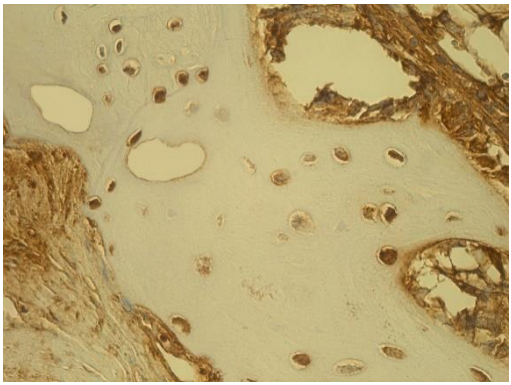

Fig. 2.13 **Moderate number of *MMP-2* positive osteocytes in the operated leg bone of *Sr-HA$_{30}$/TCP$_{70}$* rabbit. *MMP-2 IMH*, × 400**

On average, moderate number (++) of *MMP-2* positive structures was found in the tissue of **group B** *HA$_{30}$/TCP$_{70}$* operated leg. On average, few to moderate number (+/++) of *MMP-2* positive structures were observed in the non-operated leg. **Statistically significantly more** *MMP-2* positive structures were obtained in the operated leg than in the non-operated leg (U = 1.50; p = 0.002). **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the control group and operated leg (U = 34.00; p = 0.917).

The average number of *MMP-2* positive bone cells was moderate to numerous (++/+++) in the tissue samples of **group C** *Sr-HA$_{70}$/TCP$_{30}$* operated

leg. Overall, few to moderate number (+/++) of *MMP-2* positive cells were observed in the non-operated leg. **Statistically significantly more** *MMP-2* positive structures were obtained in the operated leg than in the non-operated leg (U = 0.50; p = 0.004). **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the control group and operated leg (U = 14.00; p = 0.071).

Overall, moderate to numerous (++/+++) *MMP-2* positive structures were obtained in the tissue of **group C** HA_{70}/TCP_{30} operated leg. On average, moderate number (++) of *MMP-2* positive cells was observed in the non-operated leg. **Statistically significantly more** *MMP-2* positive structures were obtained in the operated leg than in the non-operated leg (U = 6.00; p = 0.004). **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the control group and operated leg (U = 22.50; p = 0.108).

Overall, moderate number (++) of *MMP-2* positive structures was obtained in the tissue samples of **group D** *sham* operated leg (see Fig. 2.14). On average, few to moderate number (+/++) of *MMP-2* positive cells were found in the non-operated leg. **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the operated and non-operated leg (U = 9.00; p = 0.145). **Statistically significant difference was not found** between the number of *MMP-2* positive structures in the control group and operated leg (U = 23.50; p = 0.470).

Statistically significantly more *MMP-2* positive bone cells were observed after the implantation of ***Sr-HA₇₀/TCP₃₀*** granules compared to the *Sr-HA₃₀/TCP₇₀* (U = 6.5; p = 0.033) or *HA₃₀/TCP₇₀* (U = 7.5; p = 0.04) granules.

Statistically significantly more *MMP-2* positive bone cells were observed after the implantation of ***HA₇₀/TCP₃₀*** granules compared to the *Sr-HA₃₀/TCP₇₀* granules (U = 10.5; p = 0.035).

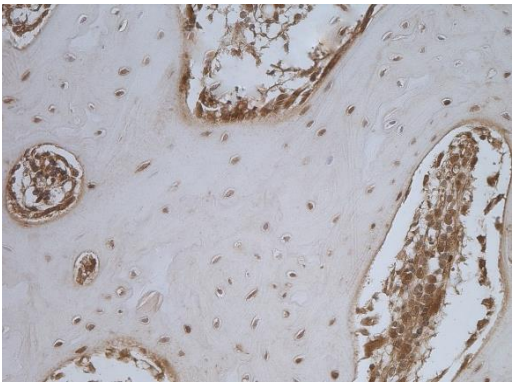

Fig. 2.14 **Moderate number of *MMP-2* positive osteocytes in the operated leg bone of *sham* rabbit. *MMP-2 IMH*, × 200**

Overall, moderate to numerous (++/+++) ***TIMP-2*** positive cells were obtained in the tissue of control or **group A.**

On average, numerous (+++) *TIMP-2* positive bone cells were obtained in the tissue samples of **group B** *Sr-HA_{30}/TCP_{70}* operated leg. Non-operated leg contained numerous (+++) *TIMP-2* positive cells. **Statistically significant difference was not found** between the number of *TIMP-2* positive structures in the operated and non-operated leg (U = 16.50; p = 0.244). **Statistically significantly more** *TIMP-2* positive structures were obtained in the operated leg compared to the control group (U = 6.00; p = 0.002).

On average, numerous (+++) *TIMP-2* positive structures were obtained in the tissue of **group B *HA_{30}/TCP_{70}*** operated leg. Overall, moderate to numerous (++/+++) *TIMP-2* positive cells were observed in the non-operated leg. **Statistically significant difference was not found** between the number of *TIMP-2* positive structures in the operated and non-operated leg (U = 11.50; p = 0.062). **Statistically significantly more** *TIMP-2* positive structures were obtained in the operated leg compared to the control group (U = 7.00; p = 0.004).

On average, numerous (+++) *TIMP-2* positive bone cells were obtained in the tissue samples of **group C** *Sr-HA_{70}/TCP_{30}* operated leg. Non-operated leg, overall, contained moderate number (++) of *TIMP-2* positive cells. **Statistically significantly more** *TIMP-2* positive structures were obtained in the operated leg than in the non-operated leg (U= 1.50; p = 0.005). **Statistically significantly more** *TIMP-2* positive structures were obtained in the operated leg compared to the control group (U = 3.00; p = 0.002).

On average, numerous (+++) *TIMP-2* positive structures were obtained in the tissue of **group C** *HA_{70}/TCP_{30}* operated leg (see Fig. 2.15). Overall, the tissue samples of non-operated leg contained moderate number (++) of *TIMP-2* positive cells. **Statistically significantly more** *TIMP-2* positive structures were obtained in the operated leg than in the non-operated leg (U = 1.50; p = 0.001). Operated leg contained **statistically significantly less** *TIMP-2* positive structures compared to the control group (U = 9.00; p = 0.003).

On average, numerous (+++) *TIMP-2* positive structures were obtained in the tissue samples of **group D** *sham* operated leg (see Fig. 2.16). On average, numerous (+++) *TIMP-2* positive cells were also found in the non-operated leg. **Statistically significant difference was not found** between the number of *TIMP-2* positive structures in the operated and non-operated leg (U = 15.50; p = 0.598). **Statistically significantly more** *TIMP-2* positive structures were obtained in the operated leg compared to the control group (U = 7.00; p = 0.01).

The presence of ***TIMP-2*** was similar in the operated leg tissue of all osteoporotic rabbits, and the **statistically significant difference was not found**.

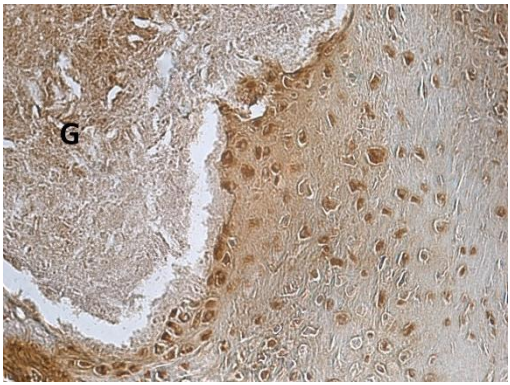

Fig. 2.15 **Numerous *TIMP-2* positive osteocytes in the operated leg bone of HA_{70}/TCP_{30} rabbit. G – granule. *TIMP-2 IMH*, × 200**

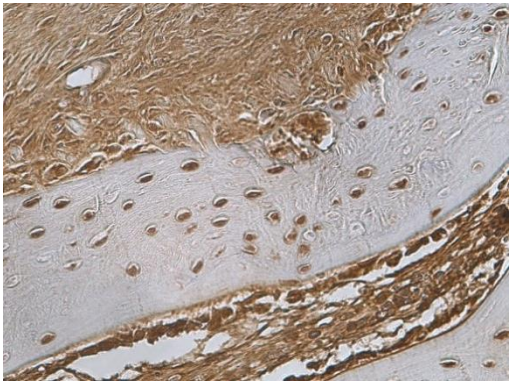

Fig. 2.16 **Numerous *TIMP-2* positive osteocytes in the operated leg bone of *sham* rabbit. *TIMP-2 IMH*, × 250**

2.3.4 Bone resorption and local immunity related factors

Overall, occasional (0/+) ***Il-1*** positive cells were observed in the tissue of control or **group A**.

On average, few (+) *Il-1* positive bone cells were observed in the tissue samples of **group B** *Sr-*HA_{30}/TCP_{70} operated leg (see Fig. 2.17). Non-operated

leg, on average, contained occasional (0/+) *Il-1* positive cells. **Statistically significantly more** *Il-1* positive structures were obtained in the operated leg than in the non-operated leg (U = 7.50; p = 0.022). **Statistically significantly more** *Il-1* positive structures were obtained in the operated leg compared to the control group (U = 12.00, p = 0.016).

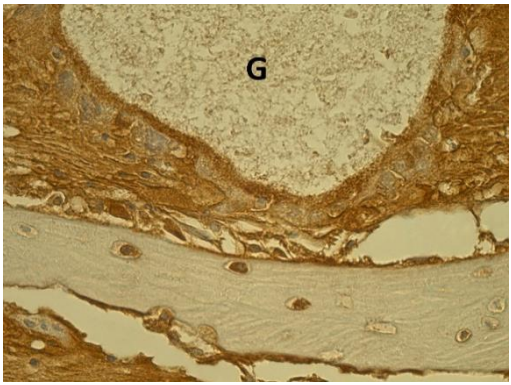

Fig. 2.17 **Few *Il-1* positive osteocytes in the operated leg bone of *Sr-HA$_{30}$/TCP$_{70}$* rabbit. G – granule. *Il-1 IMH*, × 400**

On average, few (+) *Il-1* positive structures were observed in the tissue of **group B** *HA$_{30}$/TCP$_{70}$* operated leg. On average, occasional (0/+) *Il-1* positive cells were observed in the non-operated leg. **Statistically significant difference was not found** between the number of *Il-1* positive structures in the operated and non-operated leg (U = 11.00; p = 0.071). **Statistically significantly more** *Il-1* positive structures were obtained in the operated leg compared to the control group (U = 14.00; p = 0.032).

On average, few to moderate number (+/++) of *Il-1* positive bone cells were obtained in the tissue samples of **group C** *Sr-HA$_{70}$/TCP$_{30}$* operated leg. Only occasional (0/+) *Il-1* positive cells were observed in the non-operated leg. **Statistically significantly more** *Il-1* positive structures were obtained in the

operated leg than in the non-operated leg (U = 4.50; p = 0.024). **Statistically significantly more** *Il-1* positive structures were found in the operated leg compared to the control group (U = 3.00; p = 0.002).

The highest presence of *Il-1* positive cells was observed in the tissue of **group C** HA_{70}/TCP_{30} operated leg. On average, moderate number (++) of *Il-1* positive cells was found. Non-operated leg on average contained occasional (0/+) *Il-1* positive cells. **Statistically significantly more** *Il-1* positive structures were obtained in the operated leg than in the non-operated leg (U = 1.50; p = 0.001). **Statistically significantly more** *Il-1* positive structures were found in the operated leg compared to the control group (U = 0.50; p < 0.001).

On average, few (+) *Il-1* positive structures were obtained in the tissue samples of **group D** *sham* operated leg (see Fig. 2.18). Non-operated leg on average contained few (+) *Il-1* positive cells. **Statistically significant difference was not found** between the number of *Il-1* positive structures in the operated and non-operated leg (U = 14.00; p = 0.498). **Statistically significantly more** *Il-1* positive structures were found in the operated leg compared to the control group (U = 14.50; p = 0.023).

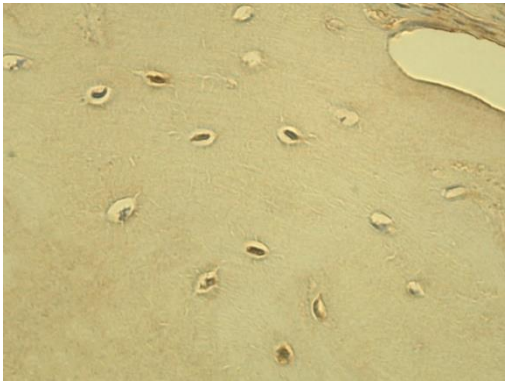

Fig. 2.18 **Few *Il-1* positive osteocytes in the operated leg bone of *sham* rabbit. *Il-1 IMH*, × 400**

Statistically significantly more *Il-1* positive bone cells were observed after the implantation of ***HA_{70}/TCP_{30}*** granules compared to the *Sr-HA_{30}/TCP_{70}* (U = 8.5; p = 0.018) or *HA_{30}/TCP_{70}* (U = 11.00; p = 0.037) granules or to the *sham* surgery (U = 5.00; p = 0.008).

Overall, moderate number (++) of ***Il-10*** positive cells was obtained in the tissue of control or **group A**.

On average, moderate number (++) of *Il-10* positive bone cells was obtained in the tissue samples of **group** B *Sr-HA_{30}/TCP_{70}* operated leg (see Fig. 2.19). Non-operated leg, on average, contained few to moderate number (+/++) of *Il-10* positive cells. **Statistically significant difference was not found** between the number of *Il-10* positive structures in the operated and non-operated leg (U = 16.00; p = 0.267). **Statistically significant difference was not found** between the number of *Il-10* positive structures in the control group and operated leg (U = 26.00; p = 0.355).

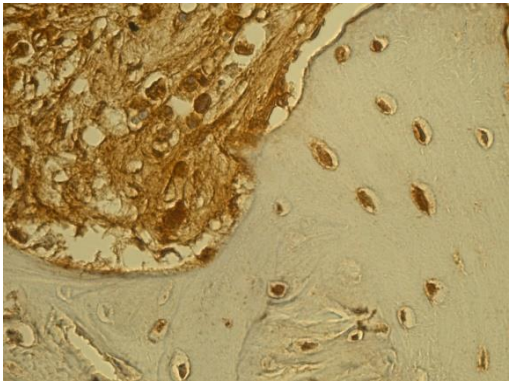

Fig. 2.19 **Moderate number of *Il-10* positive osteocytes in the operated leg bone of *Sr-HA_{30}/TCP_{70}* rabbit. *Il-10 IMH*, × 400**

Overall, moderate number (++) of *Il-10* positive structures was obtained in the tissue of **group B** HA_{30}/TCP_{70} operated leg (see Fig. 2.20). Non-operated leg, on average, contained moderate number (++) of *Il-10* positive cells. **Statistically significant difference was not found** between the number of *Il-10* positive structures in the operated and non-operated leg (U = 12.50; p = 0.089). **Statistically significant difference was not found** between the number of *Il-10* positive structures in the control group and operated leg (U = 21.50; p = 0.161).

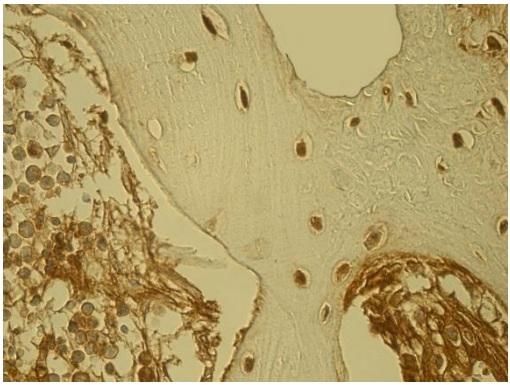

Fig. 2.20 **Moderate number of *Il-10* positive osteocytes in the operated leg bone of HA_{30}/TCP_{70} rabbit. *Il-10 IMH*, × 400**

Overall, moderate to numerous (++/+++) *Il-10* positive bone cells were obtained in the tissue samples of **group C** *Sr-*HA_{70}/TCP_{30} operated leg. Non-operated leg, on average, contained few to moderate number (+/++) of *Il-10* positive cells. **Statistically significantly more** *Il-10* positive structures were obtained in the operated leg than in the non-operated leg (U = 1.00; p = 0.005). **Statistically significant difference was not found** between the number of *Il-10* positive structures in the control group and operated leg (U = 14.50; p = 0.076).

On average, moderate to numerous (++/+++) *Il-10* positive structures were obtained in the tissue of **group C** HA_{70}/TCP_{30} operated leg. Non-operated

leg, on average, contained moderate number (++) of *Il-10* positive cells. **Statistically significantly more** *Il-10* positive structures were obtained in the operated leg than in the non-operated leg (U = 10.00; p = 0.016). **Statistically significantly more** *Il-10* positive structures were found in the operated leg compared to the control group (U = 16.00; p = 0.025).

On average, moderate to numerous (++/+++) *Il-10* positive structures were observed in the tissue samples of **group D** *sham* operated leg. Non-operated leg, on average, contained moderate number (++) of *Il-10* positive cells. **Statistically significant difference was not found** between the number of *Il-10* positive structures in the operated and non-operated leg (U = 9.50; p = 0.157). **Statistically significant difference was not found** between the number of *Il-10* positive structures in the control group and operated leg (U = 14.50; p = 0.084).

The presence of ***Il-10*** was similar in the operated leg tissue of all osteoporotic rabbits and the **statistically significant difference was not found**.

2.4 Correlation data of immunohistochemically analysed factors

Statistically significant correlations are shown in the Table 2.2. From the analysed parameters, only the tissue samples after the implantation of *Sr-HA_{70}/TCP_{30}* granules did not show the statistically significant correlations.

Table 2.2

Statistically significant positive correlations between the immunohistochemically analysed markers

Group	Factors	Correlation	Coefficient	p value
Group A				
Controls	*BMP-2/4* and *Col-1α*	VSP	$r_s = 0.829$	$p = 0.003$
	OC and *BMP 2/4*	SP	$r_s = 0.634$	$p = 0.049$
	MMP-2 and *IL-10*	SP	$r_s = 0.650$	$p = 0.042$
	BTA and *NFkB-105*	SP	$r_s = 0.657$	$p = 0.039$
Group B				
Sr-HA$_{30}$/TCP$_{70}$	*NFkB-105* and *Col-1α*	SP	$r_s = 0.734$	$p = 0.046$
	BTA and *Col-1α*	VSP	$r_s = 0.809$	$p = 0.028$
HA$_{30}$/TCP$_{70}$	*OC* and *NFkB-105*	VSP	$r_s = 0.833$	$p = 0.020$
	OC and *IL-1*	SP	$r_s = 0.772$	$p = 0.042$
	NFkB-105 and *Il-1*	VSP	$r_s = 0.814$	$p = 0.026$
	Col-1α and *Il-1*	VSP	$r_s = 0.814$	$p = 0.026$
	NFkB-105 and *Col-1α*	SP	$r_s = 0.767$	$p = 0.044$
Group C				
Sr-HA$_{70}$/TCP$_{30}$	-	-	-	-
HA$_{70}$/TCP$_{30}$	*OC* and *MMP-2*	SP	$r_s = 0.736$	$p = 0.037$
Group D				
Sham	*NFkB-105* and *MMP-2*	VSP	$r_s = 0.939$	$p = 0.005$
	NFkB-105 and *Il-1*	VSP	$r_s = 0.833$	$p = 0.039$

Abbreviations: *Sr* – strontium, *HA* – hydroxyapatite, *TCP* – tricalcium phosphate, *BTA* – bone trabecular area, *SP* – strong positive correlation; *VSP* – very strong positive correlation.

3 Discussion

To overcome and facilitate bone regeneration, several attempts are made to develop an ideal bone substitutive material enriched with biologically active molecules that improve bone remodelling and enhance new bone formation. Positive effects of *Sr* on bone regeneration has led to its application in variety of new biomaterials. It is due to the *Sr* ability to simultaneously stimulate proliferation of preosteoblastic cells and osteogenesis, while inhibiting osteoclastogenesis and bone resorption (Xie et al., 2018). However, there is a lack of studies on osteoporotic animal with *Sr* enriched biomaterials. Besides, many articles represent comparison only with two or three experimental groups. Whereas, in our study bone tissue samples were obtained from 11 different bone condition groups – biphasic calcium phosphate (*HA* and *TCP*) ceramic biomaterials in different mass ratio with and without *Sr* ions were implanted in osteoporotic female rabbits and were compared with tissue samples from healthy non-operated animals, *sham* surgery bone samples, as well as bone samples from non-operated leg. Moreover, a relative amount of *OPG, OC, NFkB-105, BMP-2/4, Col-1α, MMP-2, TIMP-2, Il-1* and *Il-10* immunopositive bone cells we studied. In this topic, the number of analysed factors that regulate bone regeneration is comparatively higher to other studies. It is worth mention-ing that scientific studies on osteoporotic rabbits after *Sr* enriched biomaterial implantation are rarely found in literature; therefore, the obtained data is compared with studies were *Sr* enriched biomaterials were implanted in only healthy rabbits or in other species. In a systematic review, *Neves et al.* included 27 *in vivo* original studies where at least two groups with or without *Sr* enriched biomaterials were used. Only nine studies were performed on rabbits, but none was conducted on osteoporotic rabbits. In addition, all studies used a different type of animal model, biomaterials and evaluation methods (Neves et al., 2017).

In the present study, bone trabecular area of healthy rabbits was 0.393 mm^2, while in osteoporotic rabbits 12 weeks after surgery it varied only from 0.206 mm^2 to 0.242 mm^2, which was statistically significant. The obtained results confirm experimentally induced osteoporosis of all operated animals. Also, bone morphological structure of all osteoporotic rabbits was with reduced compact and spongy bone compared to healthy rabbits. Thus, the results totally correspond to the findings of other authors (Sadiq, Alfaris and Allasadi, 2016). Nonetheless, the trabecular bone area was similar between all osteoporotic animals after biomaterial implantation or *sham* surgery without statistically significant outcomes. Most of the previous studies represent higher amount of new bone after *Sr* enriched biomaterial implantation compared to control tissue after pure biomaterials. Although the obtained results were found in healthy animals or in other pathological bone condition, none were conducted using experimental osteoporosis model (Tian et al., 2009; Xie et al., 2012; Tarafder et al., 2013; Zhang et al., 2013; Kang et al., 2015). Interesting results on osteoporotic rats after *Sr* enriched biomaterials were demonstrated by *Baier et al.*, where similar bone volume compared to pure biomaterials was found 12 weeks after implantation, which match the presented results, but in 24 weeks *Sr* bone samples showed significant increase of new bone (Baier et al., 2013). Conversely, *Lin et al.* showed a higher amount of new bone in three weeks after implantation of *Sr* and titanium incorporated biomaterials in osteoporotic rabbits' bone. Whereas, already in 6 weeks the bone area was similar to control group of pure biomaterial (Lin et al., 2019). These findings are supported by study results presented by *Ni et al*., where hip joint arthroplasty with *Sr* enriched *HA* bone cement was used in healthy goats. Operated animals were observed for 9 months, which is the longest observational time in the field of *Sr* enriched biomaterial sciences found in literature.

The authors concluded that the presence of *Sr* significantly improved new bone formation, better bone to implant contact and increased mechanical stability, while the capsule of fibrous tissue was not observed (Ni et al., 2006). The presented results together with the published data by other authors provide evidence that bone regeneration capacity after *Sr* enriched biomaterials is contingent on observational time after implantation. A noticeable increase of new bone is found in first weeks after implantation with following slowdown of bone formation until 6 to 12 weeks, where the bone area is similar to pure biomaterial induced bone regeneration. Then again, improvements of osteo-genesis are detected until and after 24 weeks using *Sr* enriched biomaterials. This could be explained that osteoporosis directly affects new bone formation, which is dependent on interaction between tissue and biomaterial, biomaterial degradation process and dissolution rate of *Sr* ions during the implantation period, which improves the potential of osteogenesis in a way of anabolic bone metabolism. Prolonged time of the experiment could be necessary analysing osteoporotic bone, which obviously is associated with biomaterial abilities to improve bone regenerative properties. In the present study, large sized osteoclasts were found around implanted granules, which coincide with other studies on *Sr* enriched biomaterial implantation (Cardemil et al., 2013; Kaygil et al., 2015). *Zhu et al.* demonstrated that high number of large sized osteoclasts were found around new bone and implanted biomaterials either with or without the presence of *Sr* ions (Zhu et al., 2016). Although controversial data are presented in literature about the importance of osteoclasts found in peri-implant area, this intends to state that presence of osteoclasts is associated with bone resorptive function together with osteoclast mediated biomaterial degradation process.

Collagen 1 alpha (*Col-1α*) is a major protein of extracellular bone matrix, which is secreted by osteoblasts during new bone formation and gives evidence of early bone mineralization (Ferreira et al., 2012; Nair et al., 2013). In the

present study, control bone samples showed only few to moderate number of *Col-1α* positive structures, which was statistically significantly less compared to the operated osteoporotic animals, where a moderate number of *Col-1α* was found. While non-operated bone samples of osteoporotic animals showed a moderate number of *Col-1α* positive bone cells. Scientific demonstration has shown that higher rate of bone remodelling with increased collagen synthesis is found in osteoporotic bone. However, synthetised collagen fibrils are in a smaller diameter, weaker reciprocal linking and reduced overall quality (Viguet-Carrin, Garnero and Delmas, 2006). According to the obtained data, partial agreement to such statement can be expressed, because the number of *Col-1α* positive structures in the control group and non-operated bone samples was without statistical significance. Unlike, the present study demon-strated that the expression of *Col-1α* is improved either by biomaterials or *sham* surgery in osteoporotic bone, although statistical significance was not obtained between the groups. Apart from this, *Tian et al.* showed that *Sr* enriched calcium polyphosphate biomaterials increase *Col-1α* expression compared to pure biomaterials, still it was demonstrated on healthy rabbits (Tian et al., 2009). These findings suggest that surgery alone can also improve collagen synthesis and bone regenerative properties in osteoporotic bone. It matches with the previously published data that presence of collagen increases osteoblast activity and osteogenesis (Nair et al., 2013). Particularly, *Ferreira et al.* speculated that collagen synthesis and structural singularities could be regulated with different biomaterials (Ferreira et al., 2012). Nevertheless, there are no published studies found in literature demonstrating *Sr* ability to affect collagen synthesis.

In the present study, bone samples from the control group showed a strong positive correlation between *Col-1α* and *BMP-2/4*, which suggests a strong simultaneous interaction of both factors during new bone formation. It is supported by regulatory mechanisms provided by the bone morphogenetic

proteins, where they can initiate intracellular signaling pathways from mesenchymal stem cells to osteoblast proliferation with the following osteogenesis and collagen synthesis (Jing et al., 2016). Whereas, HA_{30}/TCP_{70} bone samples showed very strong positive correlation between *Col-1α* and *Il-1*. Importantly, *Il-1* is one of the most relevant pro-inflammatory cytokines, which initiates osteoclast differentiation and bone resorption. Whereas, *Lange et al.* found that function of *Il-1* can be regulated according to the homeostatic conditions and it even can stimulate the osteoblastogenesis (Lange et al., 2010). This suggests that during bone regeneration *Il-1* can participate in regulation of osteoblasts and collagen synthesis.

The results of the current study showed that numerous **osteocalcin** (*OC*) positive bone cells were found in control tissue, which was statistically significantly more compared to only *sham* surgery samples. This finding indicates that a high level of factor related to bone mineralisation is found in the healthy tissue, whose expression is regulated also by surgery or by biomaterials to improve bone remodelling, because even higher number of *OC* positive bone cells were found in the tissue after biomaterial implantation. Numerous to abundant *OC* positive bone cells were found after $Sr\text{-}HA_{70}/TCP_{30}$ implantation, which was significantly higher than in the control tissue or after $Sr\text{-}HA_{30}/TCP_{70}$, HA_{30}/TCP_{70} and *sham* surgeries. The operated bone samples after $Sr\text{-}HA_{70}/TCP_{30}$, HA_{70}/TCP_{30} and $Sr\text{-}HA_{30}/TCP_{70}$ biomaterial implantations showed higher number of *OC* positive cells compared to non-operated bone samples. Unlike in *sham* group, both legs showed similar results. This suggests that the presence of biomaterials is important to improve the expression of *OC* in osteoporotic bone. Insights of unique properties of *S*r have been found during bone mineralisation, especially in a phase of skeletal development (Pasqualetti, Panfi and Mariotti, 2012). The findings partially match the results presented by others, where *Thormann et al.* demonstrated that a higher expression of *OC* was found in osteoporotic rats'

femur after implantation of *Sr* enriched calcium polyphosphate ceramics compared to pure biomaterial (Thormann et al., 2013). Whereas, *Tarafder et al*. showed a higher expression of *OC* in healthy rabbits' femur after implantation of *Sr* enriched *TCP* biomaterial compared to *sham* surgery (Tarafder et al., 2013). Interesting results were presented by *Wornham et al.*, where they found that the presence of pure *Sr* provokes opposite effects and inhibits osteoblast proliferation and bone mineralisation. In this *in vitro* study *Sr* salts were added to the rat calvariae osteoblast and osteoclast forming cell cultures (Wornham et al., 2014). Positive effects of *Sr* are observed only when it is together with *Ca* containing biomaterials, because osteoblastogenesis is regulated by *Sr* ability to interact through *Ca* sensitive receptors (Chattopadhyay et al., 2007). Consequently, functional properties of *Sr* are dependent on different *Ca* concentrations within biomaterials. This was supported by *Xia et al.*, where they demonstrated *in vivo* and *in vitro* studies that higher concentration of *Ca* ions improves better actions of *Sr* on higher activity of osteoblasts and new bone formation. The study was conducted using *Sr* enriched CPC biomaterials on healthy rabbits (Xie et al., 2018). In the present study, the highest number of *OC* positive bone cells were found after *Sr-HA_{70}/TCP_{30}* implantation. This could be explained that a higher proportion of *HA* provides a slower degradation of biomaterial, better affinity and higher concentration of *Ca* ions, which improves positive effects of *Sr* ions via bone remodelling process. Additionally, a higher proportion of *HA* improves involvement of Ca ions and though better affinity of *OC* and mineralisation (Rodrigues et al., 2012).

A strong positive correlation was detected between *OC* and *BMP-2/4* in the control tissue, which is similar to the results presented by other authors, where *Sr* enriched biomaterials increase the expression of *OC* and *BMP-2/4* (Andersen et al., 2013; Lin et al., 2013; Jing et al., 2016). This could be explained by a functional interaction of bone morphogenetic proteins to increase new bone

formation, which is strongly associated by simultaneous bone remodelling and mineralisation. Contrary, HA_{30}/TCP_{70} bone samples showed a very strong positive correlation between *OC* and *NFkB-105* and a strong positive correlation between *OC* and *Il-1*. It is well known that *NFkB-105* initiates signaling pathways to activate osteoclast differentiation, while *Il-1* activates osteoclast mediated bone resorption (Paiva and Granjero, 2017). Both correlations could be explained by persistent bone remodelling process, where osteoblast secreted *OC* promotes bone mineralisation, but mineralised bone advances on-going remodelling by stimulating bone resorption in next bone area. Controversial data were presented by *Neve et al.*, where they suggested that *Il-1* can inhibit synthesis of *OC* (Neve, Corrado and Cantatore, 2011). Despite the established information on osteoblast function to synthetise *OC* during bone formation, *Patti et al*., suggest that *OC* can also affect osteoclast differentiation and bone resorption (Patti et al., 2013). *Booth et al*. supported these results and found that decreased osteoclast activity and bone resorption are detected in areas of low levels of *OC* concentration (Booth et al., 2013).

Nuclear factor kappa beta-105 (*NFkB-105*) is an important transcriptional factor during osteoporosis because it regulates osteoclastogenesis via *RANK/RANKL* activation and shows the level of cellular activity (Abu-Amer, 2013). In current study, the control tissue showed moderate number of *NFkB-105* positive bone cells, which was significantly higher compared to HA_{30}/TCP_{70} tissue, but significantly lower compared to *Sr-* HA_{70}/TCP_{30}, while similar to other groups. The obtained data suggest that the expression of *NFkB-105* is quite stable and is partially affected by osteoporosis or biomaterial implantation. *Sham* surgery bone samples showed similar number of *NFkB-105* positive bone cells between operated and non-operated tissue, while higher expression of *NFkB-105* was found in operated tissue after biomaterial implantation. Therefore, surgery related trauma is not determinative for

regulating expression of *NFkB-105*. Interesting results were presented by *Tan et al*., where they pointed out that higher activity of *NFkB-105* affects osteoblastogenesis via inhibition of osteoblast differentiation with following suppression of mineralisation (Tan et al., 2014). Contrary results were demonstrated by *Xiao et al*., where bone cells were subjected under radiation. They found higher activity of phosphorylation for *NFkB-105* in *ser-536* location of osteoblasts with following enhancement of production of growth factors and cytokines, which improved cell survival (Xiao et al., 2009). Whereas, *in vitro* studies on cell cultures have demonstrated that *S*r ions can interfere signal transduction of *NFkB-105* resulting in decreased osteo-clastogenesis, while improving osteoblastogenesis (Yamaguchi and Weitzmann, 2012). The obtained results only partially coincide with these statements, because a proper comparison due to the different experimental model could not be done. Moreover, higher expression of *NFkB-105* was found together with higher expression of *OC* and *BMP-2/4*, which indicate activated and ongoing osteogenesis. Biomaterials of HA_{70}/TCP_{30} with or without *Sr* showed higher expression of *NFkB-105* compared to HA_{30}/TCP_{70}. This suggests that during osteoporosis, where bone regenerative properties are low, implanted biomaterial must be with better osteoconduction and slower degradation, which can result in longer bioactivity and better dissolution of *Sr* ions, and it seems that higher concentration of *HA* can provide more qualitative osteogenesis.

Bone remodelling process is on a continued state of bone homeostasis and can be provided only by simultaneous new bone formation and bone resorption, which are more dominant actions in osteoporosis (Tong et al., 2019). It is also confirmed by our results, where strong positive correlation in control tissue was found between bone trabecular area and *NFkB-105*. In addition, strong positive correlation was found between *NFkB-105* and *Col-1α* after *Sr*-HA_{30}/TCP_{70} and HA_{30}/TCP_{70} biomaterial implantation. As the presence of collagen indicates

traces for development of *ECM* and new bone, and the presence of *NFkB-105* indicates osteoclastogenesis, it demonstrates the significant role of *NFkB-105* during bone remodelling. Furthermore, *Montalbano et al.* found that collagen can also regulate activity, proliferation and differentiation of osteoclasts and osteoblasts via complicated signaling pathways (Montalbano et al., 2018). It is well known that *NFkB-105* regulates also pro-inflammatory cytokine, especially *Il-1*, expression (Kalaitzidis and Gilmore, 2005). The obtained data demonstrated a very strong positive correlation between *NFkB-105* and *Il-1* after HA_{30}/TCP_{70} biomaterial implant-ation and *sham* surgery. Unfortunately, no information about *Sr* enriched biomaterial interaction on bone remodelling was found; therefore, the results cannot be compared to other authors' results and it must be admitted that the presented data are unique and the observed results suggest some new and previously non-described correlation and insights.

Osteoprotegerin (*OPG*) is one of the most studied factors related to cell functionality, because it can suppress osteoclastogenesis and bone resorption via inhibiting binding *RANKL* to *RANK* (Tong et al., 2019). Activity of *OPG* is important during osteporosis induced bone remodelling changes (Jimenez et al., 2019). Moderate to numerous *OPG* positive bone cells were found in healthy control rabbits, while numerous of them were found after implantation of all *Sr* enriched biomaterials. This suggests that higher activity of *OPG* is found in osteoporotic bone conditions with compounded bone resorption, and the presence of *Sr* affects osteoclast proliferation. Moreover, *Sr* enriched biomaterials showed higher expression of *OPG* compared to pure biomaterial, *sham* surgery and non-operated bone samples, where only moderate number of *OPG* positive bone cells were detected. The findings of the current study are supported by *Tan et al.*, where they demonstrated that *Sr* enriched biomaterials in osteoporotic bone can suppress osteoclastogenesis and precursor cell differentiation into osteoclasts with following bone resorption through decreased

production or *RANKL* (Tan et al., 2014). Higher activity of osteoblasts, which expresses *OPG*, were found after *Sr* enriched biomaterials even in osteoporotic bone suggesting that the presence of *Sr* can indirectly improve osteoblastogenesis (Tong et al., 2019). The results coincide with the results demonstrated before. *Thorman et al*., found higher expression of *OPG* in osteoporotic rats' femur six weeks after implantation of *Sr* enriched *CPC* biomaterials compared to pure ceramics or *sham* surgeries. The authors concluded that despite *Sr* ability to suppress osteoclastogenesis, there is no evidence that presence of *Sr* affects large sized osteoclast functionality during biomaterial degradation (Thormann et al., 2013). Similarly, large sized osteoclasts were also found in tissue samples after pure biomaterial implantation. Furthermore, higher expression of *OPG* was demonstrated in other studies after *Sr* enriched biomaterial implantation compared to pure biomaterial affected bone (Lin et al., 2013; Singh et al., 2014). No positive correlations were found between *OPG* and other factors. This could be explained, on the one hand, by the independent role of *OPG* regulating osteoclast mediated bone resorption in healthy and osteoporotic rabbits bone conditions. On the other hand, osteoclast differentiation could be regulated by another mechanism, where *OPG* can interact with transmembrane protein and its ligand through *Fas/FasL* system, which corresponds to mitochondrial caspase activity. *OPG* is able to block the receptor and initiate apoptosis of osteoclast precursors with following suppression of bone resorptive activity (Liu et al., 2015). *OPG* is considered to be the most significant tissue factor in osteoporotic bone, because of its ability to suppress osteoclastogenesis, which is very important to provide renewal of bone functionality in favour of anabolic processes during bone remodelling.

In the current study, few to moderate number of **bone morphogenetic protein-2/4** (*BMP-2/4*) positive bone cells were found in the control group, which was significantly less compared to all groups after biomaterial

implantation. Whereas, the number of *BMP-2/4* positive osteocytes was similar between *sham* surgery and control group. Interesting that operated bone samples of HA_{30}/TCP_{70} and *sham* surgery showed similar level of *BMP-2/4* positive cells compared to non-operated bone samples. Moreover, *sham* surgery bone showed the most inexpressive changes of *BMP-2/4* positive cells compared to control tissue. The obtained data suggest that the presence of biomaterials in osteoporotic bone increases higher expression of *BMP-2/4* compared to surgery alone. Although, bone trabecular area was similar between osteoporotic animals, it seems that the biomaterials provide more favourable ambiance to enhance new bone formation due to the better osteoinduction, which is controlled by *BMP-2/4*. *BMP 2/4* belongs to transforming growth factor family and regulates bone regeneration through initiating migration and differentiation of mesenchymal stem cells into preosteoblasts and improves expression of genes and different factors during osteoblastogenesis (Jang, Kim and Kim, 2012; Lin et al., 2015). The presences of *Sr* promotes osteoblast differentiation with following increase of bone morphogenetic proteins via interaction through *Ca* sensitive receptors (Tao et al., 2018). The obtained results demonstrated that the most noticeable increase of *BMP 2/4* positive bone cells was after $Sr\text{-}HA_{70}/TCP_{30}$ biomaterial implantation, and it was significantly higher compared to HA_{30}/TCP_{70} biomaterials and *sham* surgery. The results partially match with the findings presented by others. For example, *Tian et al.* observed higher expression of *BMP-2/4* after implantation of *Sr* enriched calcium polyphosphate biomaterials compared to pure biomaterials in healthy rabbits' bones (Tian et al., 2009). Whereas, *Thormann et al.* found insignificant increase of *BMP-2/4* after *Sr* enriched biomaterial implant
ation in osteoporotic rabbits (Thormann et al., 2013). It was relevant that $Sr\text{-}HA_{70}/TCP_{30}$ biomaterials showed the highest number of *BMP-2/4* positive bone cells compared to all other groups. This suggests that presence of *Sr*

improves functional meaning of *BMP-2/4* and enhances the potential of osteogenesis even in osteoporotic conditions. Particularly it was demonstrated by *Tao et al*., where faster bone healing was detected after *Sr* and *BMP-2* enriched biomaterials in osteoporotic rats (Tao et al., 2018).

Above mentioned correlations between *BMP-2/4*, *Col-1α* and *OC* are associated with enhanced bone cell activity, which gives evidences on bone regeneration in osteoporotic bone conditions. Similar correlations were presented by others, where higher expression of *BMP-2/4* was found together with increased levels of *OC* and *Col-1α* (Young et al., 2008; Baier et al., 2013; Barbeck et al., 2015). It is important to emphasise that these findings were detected only in healthy rabbits. Therefore, our results suggest that biomaterials can activate osteoblastogenesis in osteoporotic rabbits' bone, which is even with higher *BMP-2/4* expression compared to healthy tissue.

Matrix metalloproteinases (*MMPs*) is responsible for degradation of *ECM* during bone remodelling phase, where osteoclast adhesion is needed for further bone resorption. As osteoclasts fail to produce collagenases, *MMPs* are indispensable to degrade non-mineralized part of the bone matrix (Liang et al., 2016). Similar expression of *MMP-2* positive bone cells was found between control rabbit and osteoporotic operated rabbit tissue samples, as well as similar expression between biomaterials and *sham* surgery. Significant results were obtained only after implantation of *Sr-*HA_{70}/TCP_{30} compared to *Sr-*HA_{30}/TCP_{70} and HA_{30}/TCP_{70} bone samples. Nonetheless, higher expression of *MMP-2* was found in operated bone samples compared to non-operated ones after HA_{30}/TCP_{70}, *Sr-*HA_{70}/TCP_{30} and HA_{70}/TCP_{30} biomaterial implantation. It can be claimed that the presence of *MMP-2* in osteoporotic bone represents bone quality properties, because it enhances resorption of *ECM* components, which afterwards are replaced with a new bone. Controversial data are presented in literature on *MMPs* expression after *Sr* enriched biomaterial implantation. On the

one hand, *Xie et al.* found that *Sr* containing calcium polyphosphate granules increase the expression of *MMP-2* on healthy rabbits, thus pointing out the significance of *Sr* during *ECM* remodelling. The authors substantiated these results by hypothesis that *Sr* enhances osteoblast transformation into osteocytes, which increase synthesis of *MMPs* (Xie et al., 2012). On the other hand, *Braux et al*. demonstrated that *Sr* enriched biphasic *CPC* showed lowered levels of *MMP-2* compared to pure biomaterials (Braux et al., 2011). The obtained results, although on osteoporotic rabbits, partially match the previously demonstrated findings. It was found that biomaterials affect remodelling of *ECM* in osteoporotic bone similar to the level of healthy bone. Moreover, an increase of positive *MMP-2* bone cells was found in operated bone compared to non-operated bone samples. It should be noted again that there is no conformity of opinion on functional importance of *MMP-2*. Everyone agrees that *MMP-2* is a relevant tissue degrading factor during bone remodelling, while others speculate that high levels of *MMP-2* with following bone turnover process can provoke metal implant dislocation and increase the risk of pathological bone fractures due to increased bone resorption. Altogether it is suggested to evaluate the levels of *MMPs* together with the expression levels and interrelation of their inhibitors in normal and pathological bone conditions (Braux et al., 2011; Liang et al., 2016).

In the present study, bone samples from control group showed strong positive correlation between *MMP-2* and *Il-10*, which confirms previous findings that *Il-10* is an important regulatory cytokine during bone remodelling, where it regulates expression of *MMPs* (Jung et al., 2013). Whereas, HA_{70}/TCP_{30} bone samples demonstrated strong positive correlation between *OC* and *MMP-2*, which is supported by the fact that in *MMPs* deficit bone low mineralisation levels are found, which affects mechanical stability and increase the risk for pathological fractures (Alliston, 2014). Besides, the bone tissue from *sham* surgery group showed very strong correlation between *MMP-2* and *NFkB-105*.

It could be explained by ongoing bone matrix remodelling process and bone resorption, which is regulated by *NFkB-105* induced osteo-clastogenesis. Moreover, it is well known that *Il-1* participates in regulatory mechanisms for secretion of *MMP-2* (Laquerriere et al., 2004). It is also supported by the obtained data, where significant correlations were found between *MMP-2*, *Il-10* and *NFkB-105*. The last two are involved in regulation of osteoclast differentiation and synthesis of pro-inflammatory cytokines, confirming *MMP-2* role regulating other biologically active molecules to maintain normal bone homeostasis.

Tissue inhibitor of matrix metalloproteinase-2 (*TIMP-2*) belongs to the multifunctional protein family, which is responsible for inhibiting the activity of *MMP-2*. The importance of *TIMP-2* is demonstrated during the development phase of skeleton, where hyper degradation of *ECM* can lead to skeletal anomalies (Liang et al., 2016). Significant results of *TIMP-2* positive bone cells were found between control and osteoporotic animals. Operated rabbit bone samples showed higher amount of *TIMP-2* positive bone cells, although the expression level of factor was similar between biomaterials and *sham* surgery bone. Only *Sr-HA_{70}/TCP_{30}* and *HA_{70}/TCP_{30}* bone samples showed significantly higher amount of *TIMP-2* in operated leg compared to non-operated leg. The results could be explained by the fact that higher activity of *TIMP-2* is found during bone regeneration, especially in the phase of mineralisation, where secretion of *TIMP-2* is regulated by matured osteoblasts. Moreover, *TIMP-2* can also regulate activity of osteoclasts during bone resorption (Paiva and Granjero, 2017). Altogether, despite the osteoporosis, it shows enhanced bone regeneration after biomaterial implantation and surgery. Furthermore, it indirectly shows the *TIMP-2* regulatory function on osteoclast activity, which is known to be much higher in osteoporotic bone. It is supported by *in vitro* study, where *TIMP-2* regulates osteoclast mediated bone resorption (Sobue et al., 2001). It is also substantiated by *in vivo* studies, where *MMPs* inhibitor deficit bone and cartilage

represents with distinct bone erosions and degradations features (Sahebjam, Khokha and Mort, 2007).

In current study, similar amount of *TIMP-2* positive bone cells was detected in *Sr* enriched and pure biomaterial bone samples. Whereas, *Braux et al*. demonstrated higher expression of *TIMP-2* after *Sr* enriched *CPC* biomaterial implantation compared to pure one (Braux et al., 2011). It should be noted that this was an *in vitro* study on extracted human osteoblasti cell culture. The results showed no significant correlation between *TIMP-2* and other analysed factors. However, the association with *MMP-2* must be addressed. Overall, higher levels of *TIMP-2* positive bone cells were found compared to *MMP-2* among all study groups. This could be explained by the fact that *TIMP-2* has some additional functional implication on bone cells and bone remodelling process. The obtained data on expression levels of *TIMP-2* are unique, because there is lack of information about *Sr* enriched biomaterial interaction *in vivo* and *TIMP-2*.

The current study demonstrated significantly higher **interleukin-1** (*Il-1*) positive bone cells in osteoporotic operated bone samples compared to the control group. The control group showed only a few *Il-1* positive bone cells, while in osteoporotic rabbits few to moderate number of *Il-1* positive bone cells were found. Bone samples from *Sr-*HA_{30}/TCP_{70}, *Sr-*HA_{70}/TCP_{30} and HA_{70}/TCP_{30} operated leg showed higher expression of *Il-1* positive bone cells compared to non-operated leg, while no difference was found in *sham* surgery group. HA_{70}/TCP_{30} bone samples showed the highest number of *Il-1* positive bone cells, which coincide with results presented by *Ninomiya et al*., where they proved that *HA* and *HA/TCP* biomaterials increase the level of pro-inflammatory cytokine *Il-1* with following activation of bone resorption, which, unfortunately, in a long term can lead to intensified osteolysis and metal implant dislocations (Ninomiya et al., 2001). *Il-1* is the most studied pro-inflammatory cytokine, which participates in regulatory function of tissue homeostasis. Especially, taking part

in osteoporotic bone resorption, where it activates osteoclast differentiation and promotes their function (Lee et al., 2010). Nevertheless, some positive effects of *Il-1* on bone remodelling have been presented. For example, in osteoporotic bone *Raisz et al*. found that higher expression of *Il-1* after bone fracture fixation improves bone remodelling even in osteoporotic bone (Raisz, 2005). The obtained results are supported by this finding, too. In current study, all operated rabbits showed higher expression of *Il-1*, even after *Sr* enriched biomaterial implantation. Whereas, *Fernandez et al*. demonstrated that presence of *Sr* can decrease the production of *Il-1* (Fernandez et al., 2013). This could be explained by the ability of *Sr* to increase the expression of *OPG* with following suppression of *RANK/RANKL* signaling pathway and osteoclastogenesis. In the present study, the highest number of *Il-1* positive bone cells were found after HA_{70}/TCP_{30} implantation compared to $Sr\text{-}HA_{30}/TCP_{70}$ bone samples, whereas higher number of *OPG* was detected in all bone samples after *Sr* enriched biomaterial implantation than after pure biomaterials. Interestingly, control group and non-operated bone samples from osteoporotic rabbits showed similar number of *Il-1* positive bone cells even though rabbit have osteoporosis. Based on this, it can be speculated that after 12 weeks of osteoporosis induced bone remodelling changes and following surgery-initiated bone regenerative properties, osteoporosis could have achieved the plateau phase, where bone resorptive process is similar to healthy rabbits. However, it must pointed out that bone trabecular area in healthy rabbit was almost twice bigger. It could be important to analyse bone remodelling related factors in different time periods after implantation or *sham* surgery. *Vamze et al*. have investigated pro and anti-inflammatory cytokine expression in healthy rabbits' mandible after 3, 4.5, 6 and 8 months after implantation of different *CPC* biomaterial. The authors demonstrated that the most active expression of *Il-1* was found 3 months after surgery, while deficiency of the factor was detected in a 4.5-month time. Only for 6 to 8 months after

implantation, the authors observed reiterative but slow increase of *Il-1*. Inconstancy of *Il-1* could be explained as a responsible reaction to surgery related tissue trauma and biomaterial itself. Whereas, the increase of factor could be associated with gradual slowdown of latent inflammation (Vamze, Pilmane and Skagers, 2012).

It is import to emphasise that a similar number of *Il-1* positive bone cells were detected between operated and non-operated tissue after *Sr* enriched biomaterial implantation compared to other groups. These results suggest that the presence of *Sr* does not increase either the local or generalised tissue inflammotry reactions. This coincides with the data presented by *Tie et al*., in which no morphological changes of thigh muscles, as well as no signs of necrotic tissue or no inflammatory infiltrates in spleen, kidney or liver were detected after *Sr* enriched biomaterial implantation (Tie et al., 2016). This is also supported by *Baier et al*., where normal concentration of *Sr* was detected in peripheral blood after *Sr* enriched *CPC* biomaterial implantation (Baier et al., 2013). Overall, local use of *Sr* together with different biomaterials is safe, effective and promising technology, which could avoid negative effects associated with systematic use of *Sr* containing medication. The presented data by *Neve et al*. demonstrated that production of *Il-1* significantly decreases expression of *Col-1* and *OC* in bone tissue (Neve, Corrado and Contatore, 2011). The results of current study showed the opposite – a strong positive correlation after HA_{30}/TCP_{70} biomaterial implantation was detected between *OC* and *Il-1* and very strong positive correlation between *Col-1α* un *Il-1*. This could be explained by a significant value of inflammatory cytokine in osteoporotic bone, where intensified bone resorption could provoke better remodelling of *ECM* and new bone formation, which is stimulated by biomaterials. *Lange et al.* also approved that high levels of *Il-1* affect osteoblastogenesis during fracture healing by stimulating osteoblasti proliferation (Lange et al., 2010).

In present study, the number of **interleukin-10** (*Il-10*) positive bone cells were similar in controls and osteoporotic bone samples. *Il-10* is the most studied anti-inflammatory cytokine, whose regulative function is important during bone remodelling, where it controls resorption rate of bone matrix and synthesis of pro-inflammatory cytokines (Zhang et al., 2014). Moreover, *Il-10* is the main regulatory factor of *Il-1* expression (Schraufstatter et al., 2012). Interestingly, all bone samples of our study showed constantly higher amount of *Il-10* positive bone cells compared to *Il-1* positive bone cells. Furthermore, similar expression of *Il-10* between *sham* surgery and biomaterial bone samples confirms equal level of anti-inflammatory tissue reactions, which are stable and similar to healthy rabbits. The *in vivo* studies on genetically deleted *Il-10* represent with reduced skeleton bone mass due to the uncontrolled osteoclast differentiation and bone resorption (Zhang et al., 2014). There are several molecular mechanisms by which *Il-10* can suppress osteoclastogenesis. Principally, *Il-10* can improve synthesis of *OPG* with simultaneous inhibition of *RANKL* and macrophage colony stimulating factor expression (Liu et al., 2006). It is also important to emphasise that *in vitro* studies have shown that *Sr* enriched biomaterials decrease development of *M1* inflammatory macrophages and inhibits synthesis of tumour necrosis factor *α*, *Il-1*, *Il-6*. Simultaneously, *Sr* stimulates proliferation of *M2* macrophages and expression of *Il-10*, resulting in a more favourable environment for the biomaterial osteointegration and bone homeostasis (Li et al., 2018). Other *in vitro* and *in vivo* studies have demonstrated that *Sr* enriched biomaterials improve expression of *Il-10* 3.5 times higher compared to pure ceramics, thus proving the immunomodulatory effect of *S*r during bone remodelling (Yuan et al., 2017).

In current study, higher amount of *Il-10* positive bone cells of operated bone samples compared to non-operated ones were detected after *Sr-*HA_{70}/TCP_{30} and HA_{70}/TCP_{30} biomaterial implantation, while only HA_{70}/TCP_{30} bone samples

showed higher expression of *Il-10* compared to control group of healthy rabbits. Besides the number of *Il-10* positive bone cells after HA_{70}/TCP_{30} biomaterial implantation was the highest compared to other groups. These tissue changes could be affected by *HA* and *TCP* mass ratio, because higher concentration of *HA* provides slower degradation and solubility of biomaterials, which could be more preferable in osteoporosis. As well as slower dissolution of *Sr* ions from implanted granules could ensure more qualitative and quantitative bone remodelling changes even in osteoporotic bone, where bone regenerative properties without implantation are reduced.

The obtained results of the study could be appreciated as unique data on pure and *Sr* enriched *CPC* biomaterial implantation. For the first time 11 different bone sample groups were analysed according to one protocol and one methodology principles. Healthy bone samples were compared with osteoporotic ones after $Sr\text{-}HA_{30}/TCP_{70}$, HA_{30}/TCP_{70}, $Sr\text{-}HA_{70}/TCP_{30}$, HA_{70}/TCP_{30} biomaterial implantation or *sham* surgery, as well as bone samples from non-operated leg. Moreover, many of bone metabolism related factors as *OPG, OC, NFkB-105, BMP-2/4, Col-1α, MMP-2, TIMP-2, Il-1* and *Il-10* were evaluated. According to literature, some of the analysed factors in the field of tissue regeneration after *Sr* enriched biomaterial implantation were studied for the first time. The obtained results and correlation data of factors possess a descriptive value for further comparisons and understandings of complicated regulatory mechanisms during bone regeneration.

The osteoporotic bone samples demonstrated significant difference between the analysed factors compared to control group. Despite the lowered regenerative properties under osteoporotic conditions, the obtained results prove that tissue trauma and biomaterials are key determinative occasions to improve bone regeneration even better than in healthy tissue. Bone samples after biomaterial implantation showed higher expression of *Col-1α*, *BMP-2/4*, *TIMP-*

2 and *Il-1* compared to healthy tissue. Moreover, bone samples after *Sr-*HA_{70}/TCP_{30} biomaterial implantation improved expression of *OC* and *NFkB-105*, but HA_{70}/TCP_{30} bone samples higher expression of *Il-10*, while number or *OPG* and *MMP-2* positive bone cells were similar to control tissue. Likewise, *sham* surgery bone samples demonstrated higher expression of *Col-1α*, *TIMP-2* and *Il-1* compared to control group. Therefore, it can be maintained that in addition to surgery improved bone matrix formation, regulation and immunomodulation, the presence of biomaterials can improve osteogenesis. Whereas, the presence of *Sr* enhances bone mineralisation and cellular activity even in osteoporotic bone.

When analysing tissue reactions between operated and non-operated bone samples of osteoporotic rabbits, unique results were found that *sham* surgery induced tissue trauma can improve only the formation of bone matrix. Conversely *Sr-*HA_{70}/TCP_{30} and HA_{70}/TCP_{30} bone samples were characterised by an increased expression of *Col-1α, NFkB-105, OC, OPG, BMP-2/4, MMP-2, TIMP-2, Il-1* and *Il-10*, which provide better stimulation of *ECM* remodelling, immunomodulative activity, osteoblastogenesis and mineralisation process with following increased cellular activity and suppressed osteoclastogenesis. It should be noted that mass ratio between *HA* and *TCP* plays a significant role during bone regeneration in osteoporotic bone.

The *Sr* enriched biomaterials demonstrated better impact on osteogenesis and increased cellular activity, as well as suppressed osteoclastogenesis compared to pure biomaterials and *sham* surgery. The *Sr* enriched biphasic *CPC* within a mass ratio of HA_{70}/TCP_{30}, significantly improve bone mineralisation and *ECM* remodelling processes, while levels of pro-inflammatory and anti-inflammatory cytokines are similar to pure biomaterials or *sham* surgery, thus providing permanent, but stable immunomodulative cell activity.

Overall, the results suggest that *Sr-HA_{70}/TCP_{30}* biomaterials propose the most relevant tissue changes of osteoporotic bone and are estimated as innovative and some of them even as unique.

Conclusions

1. Bone structure of healthy rabbits is characterised by distinct compact and spongy bone, which is statistically bigger than is osteoporotic rabbits. Whereas, bone trabecular area between all operated animals is similar, which indicates stable level of induced osteoporosis and thus the increase of bone area is not detected in 12 weeks even after biomaterial implantation.

2. Presence of osteoclasts and giant cells around biomaterials with or without *Sr* ions indicates ongoing process of biomaterial degradation. As well as missing inflammatory reaction in tissue around *Sr* enriched biomaterials demonstrates that local application of S*r* is safe.

3. Significant increase of *Col-1α* in all osteoporotic operated tissue samples gives evidence that biomaterials and even *sham* surgery related tissue trauma indirectly regulate osteoblast activity and beginning of new bone formation by promoting synthesis of extracellular matrix proteins. Similar number of *Col-1α* positive bone cells after biomaterial implantation and *sham* surgery indicates equitable role in development of bone extracellular matrix.

4. *Sr-HA$_{70}$/TCP$_{30}$* biomaterials provoke most noticeable increase of *OC* positive bone cells compared to control and all other experimental groups, thus highlighting the role of *Sr* during mineralisation process of the new bone.

5. Significant increase of *BMP-2/4* positive bone cells in all osteoporotic bone samples after biomaterial implantation compared to control and *sham* surgery group, gives direct evidence that biomaterials intensify the processes of osteoblast differentiation and osteogenesis. Dominant increase of *BMP-2/4* positive bone cells after *Sr-HA$_{70}$/TCP$_{30}$* biomaterial implantation justifies the role of *Sr* by promoting osteoblastogenesis.

6. High number of *NFkB-105* positive bone cells together with significant correlation between *NFkB-105* and increased bone trabecular area in control group, presents high cellular activity, stable osteoclastogenesis and overall

processes of osteogenesis in healthy rabbits' bone. Whereas, increase of *NFkB-105* positive bone cells in operated leg after biomaterial implantation demonstrates the role of biomaterials to enhance bone remodelling more rapidly. Most noticeable increase of *NFkB-105* after *Sr-HA_{70}/TCP_{30}* biomaterial implantation compared to control and other experimental groups, substantiates the role of *Sr* to regulate osteoclastogenesis and bone remodelling, while increase of *NFkB-105* after *HA_{70}/TCP_{30}* biomaterial implantation indicates the role of higher concentration of the hydroxyapatite in osteoporotic bone.

7. Significant increase of *OPG* positive bone cells after *Sr* enriched biomaterial implantation gives evidence for *Sr* determinative role to suppress the osteoclastogenesis and compounded bone resorption in osteoporotic rabbits' bone, which is similar to ongoing overall osteogenesis in healthy rabbits.

8. Similar number of *MMP-2* positive bone cells in control and osteoporotic operated rabbits shows equal influence on initiated processes for degrading bone extracellular matrix. Increase of *MMP-2* positive bone cells in operated leg bone samples after biomaterial implantation, gives evidence for biomaterial local support and improvement in bone remodelling processes. Whereas, increase of *MMP-2* positive bone cells after *Sr-HA_{70}/TCP_{30}* and *HA_{70}/TCP_{30}* biomaterial implantation suggests its role for better osteo-conduction with following enhancement of cell functionality during bone matrix remodelling.

9. Significant increase of *TIMP-2* positive bone cells after biomaterial implantation and *sham* surgery is estimated as regulatory control mechanism against induced resorption of extracellular matrix components adjusted by *MMP-2*. Local increase of *TIMP-2* positive bone cells after *Sr-HA_{70}/TCP_{30}* and *HA_{70}/TCP_{30}* biomaterial implantation is associated with increased levels of *MMP-2*, thus indicating controlled balance of extracellular matrix degrading process during bone remodelling.

10. Local increase of *Il-1* positive bone cells after biomaterial implantation and *sham* surgery is evaluated as normal organism reaction to surgery related tissue trauma with subsequent activation of bone remodelling. Similar expression of *Il-1* after biomaterial implantation and *sham* surgery gives evidence that local application of *Sr* enriched biomaterials do not stimulate enhanced inflammation in peri-implant region.

11. Similar expression of *Il-10* positive bone cells in osteoporotic rabbits' bone is estimated as stable anti-inflammatory indicator after biomaterial implantation and *sham* surgery.

12. *Sr-HA_{70}/TCP_{30}* biomaterials compared to other biomaterials or *sham* surgery demonstrate most noticeable changes of osteoporotic bone tissue, which are characterised by increased expression of *Col-1α, OC, BMP-2/4, NFkB-105, OPG, MMP-2, TIMP-2, Il-1* and *Il-10*. Overall, expression of these factors highlights improved bone mineralisation, extracellular matrix remodelling and cellular activity processes compared to *Sr-HA_{30}/TCP_{70}* biomaterials.

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1.Pilmane M., Maķe K., Šalma I., Šalms G., Ločs J., **Zariņš J**. Kaulu tilpuma pētījumi veselu un osteoporozes skartu trušu kaulos pēc dažādu biomateriālu implantācijas (Eng., Bone volume studies in healthy and osteoporotic rabbit bones after implantation of various biomaterials). In: *Rīga Stradiņš University Scientific conference*, Riga, Latvia, 2017, abstract book: 42. (Poster presentation).

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