



Vladislavs Ananjevs

**Bichasic Calcium Phosphate
Bioceramic Materials Influence
on Osteoporotic Bone
Biomechanical Parameters
and Bone Density
(experimental research)**

Summary of the Doctoral Thesis
for obtaining a doctoral degree (*Ph.D.*)

Sector – Medical Engineering
Sub-sector – Biomechanics

Rīga, 2021



Vladislavs Ananjevs

ORCID 0000-0002-0498-1403

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The Doctoral Thesis was developed at Rīga Stradiņš University Biomechanical laboratory, Latvia

Supervisor of the Doctoral Thesis:

Dr. habil. med., Professor **Jānis Vētra**,

Rīga Stradiņš University, Latvia

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

Rīga Stradiņš University, Latvia

Scientific Advisor:

Dr. med., Dr. habil. med., Professor **Andrejs Skāģers**,

Rīga Stradiņš University, Latvia

Official Reviewers:

Dr. sc. ing., Associate Professor **Kristīne Šalma-Ancāne**,

Riga Technical University, Rudolfs Cimdins Biomaterials

Innovation and Development Centre, Latvia

Dr. med., Professor **Ilmārs Dūrītis**,

Latvia University of Life Sciences and Technologies

Dr. med. **Simonas Grybauskas**,

Vilnius University, Lithuania

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Secretary of the Promotion Council:

Dr. biol., Professor **Pēteris Tretjakovs**

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Abbreviations

GPa	gigapascal
HAP	hydroxyapatite
HU	Hounsfield units
LLU	Latvia University of Life Sciences and Technologies
M	molar mass
MPa	megapascal
p	level of marginal significance
Pa	pascals
RSU	Rīga Stradiņš University
SD	standard deviation
TCP	tricalcium phosphate
VPP	National research programme

Introduction

Topicality of the Thesis

Osteoporosis is a systemic skeletal disease characterised by decreased bone mass and changes in bone microarchitecture with increased bone fragility (*Blanchard et al.*, 2019). The prevalence of osteoporosis is increasing with age and survival. As society ages, osteoporosis is increasingly becoming a global epidemic. Osteoporosis currently affects more than 200 million people worldwide. In European countries, about 30 % of all postmenopausal women have osteoporosis diagnosis (*Sözen et al.*, 2017).

Prevention of osteoporosis is in reduction of fall, prevention of visual and hearing impairments, physical activity, smoking and cessation of alcohol consumption, as well as adequate intake of vitamin D, protein and calcium (*Föger-Samwald et al.*, 2020). Oral bisphosphonates (aledronate, risedronate and ibandronate) are commonly used in the medical treatment of osteoporosis. For women with oral bisphosphonates contraindication, intravenous bisphosphonates or denosumab are the best alternative (*Kanis et al.*, 2019). About 1 in 3 women and 1 in 5 men over the age of 50 experience osteoporotic fractures. Fractures caused by osteoporosis increase morbidity and premature mortality, especially in the elderly (*Sözen et al.*, 2017). Treatment of osteoporotic fractures is challenging because of the lower fracture healing capacity, which correlates with a much higher rate of implant fixation failure compared to the younger patients. Surgical procedures are often performed to repair damaged bone using screws or fixation plates. Due to the high porosity and low strength of osteoporotic bone tissue, implants are often supplemented with various bone biomaterials to improve the outcome (*Offermanns et al.*, 2018).

Since the beginning of the 21st century, the widespread use of biomaterials in orthopaedic practice has begun rapidly. Over the past decade, several segments

of the health field have sought biocompatible materials for the reconstruction of bone defects (*Ratner et al.*, 2013). Research into the possibilities of using bone tissue replacement biomaterials is also relevant in Latvia. The biomaterial must be compatible with the implantation site, non-toxic, non-carcinogenic, chemically and biologically stable, with appropriate density, weight, mechanical strength and flexibility. Autogenous bone grafts, which contain mineral components and many immunocompetent cells for osteogenesis, are the best bone replacement material; however, two surgical sites are required and the bone source is limited (*Leite et al.*, 2008).

Bone regeneration is a physiological process of bone formation that occurs during normal fracture healing and is involved in the continuous process of bone life over a lifetime. However, there are various clinical conditions that require extensive bone regeneration, such as in cases of osteoporotic bone fractures (*Dimitriou et al.*, 2011). Bioceramic materials have a huge potential for natural bone tissue regeneration (*Wang et al.*, 2017). Calcium phosphate-containing biomaterial, hydroxyapatite, which can be pure or coated with drugs, is one of the best known and clinically researched bone regeneration products. Calcium phosphate stimulates bone healing due to osteoconductivity, biocompatibility, and biodegradability properties (*Schlickewei et al.*, 2015). Biphasic calcium phosphate bioceramics, as a bone substitute, is used to improve the properties of osteoporotic bones and promote bone healing. Calcium hydroxyapatite (HAP) is highly bioaccumulative and forms a direct link to bone. Tricalcium phosphate (TCP) acts as a catalyst to stimulate new bone formation (*Ratner et al.*, 2013). Strontium-containing calcium phosphate bone cements are promising materials for the recovery of osteoporosis-related bone defects because they stimulate bone formation while limiting osteoclast function (*Lode et al.*, 2017).

Riga Technical University Rudolfs Cimdins Biomaterials Innovation and Development Centre conducts research of bone tissue replacement materials: it has more than 20 years of experience in cooperation with RSU Department of Oral and Maxillofacial Surgery in experimental and clinical approbation of new synthetic biomaterials. The work is underway on the synthesis of hydroxyapatite, biphasic calcium phosphate cement, PMMA cement, glass-ionomer cement and research of their properties. Of these, in the Author's experimental animal study, biphasic calcium phosphate bioceramic materials were used.

Any biomaterial that is implanted in a living organism causes a specific local reaction. The reaction depends on physical and chemical properties of the material. The theory of reactogenicity was developed by dividing the tissue response to implantation into non-specific and specific, which together form a biocompatibility (*Slutskii et Vetra*, 1996). There is little description in the literature of the body's overall response to biomaterial implantation. It is still unclear what changes in bone tissue properties near the implantation area and in remote areas after biomaterial implantation.

Objective of the Thesis

The aim of the study was to determine the changes in bone mineral density, biomechanical and morphological properties after local amplification of the trochanter major area of the thigh with biphasic calcium phosphate bioceramic materials in rabbits with experimental osteoporosis.

Tasks of the Thesis

1. To determine the ultimate strain of operated, non-operated thigh and mandibular bone samples for different groups of experimental animals;
2. To determine the ultimate stress of operated, non-operated thigh and mandibular bone samples for different groups of experimental animals;
3. To determine the flexure modulus of operated, non-operated thigh and mandibular bone samples for different groups of experimental animals;

4. To determine the mineral density of operated, non-operated thigh and mandibular bone samples for different groups of experimental animals;

5. To compare the biomechanical parameters and mineral density of operated and non-operated thigh samples of experimental animals after implantation of different types of biomaterials;

6. To compare the obtained results between different groups of experimental animals after implantation of biomaterials with or without strontium;

7. To determine the trabecular bone area in the cross-section of the mandibular bone samples after implantation of different types of biomaterial in the case of experimental animals and control group.

Hypothesis of the Thesis

Local enhancement with biphasic calcium phosphate bioceramic materials in the trochanter major area of the thigh improves bone biomechanical properties and increases mineral density in animals with experimental osteoporosis.

Novelty of the Thesis

It was proved that the biphasic calcium phosphate bioceramic materials of Riga Technical University Rudolfs Cimdins Biomaterials Innovation and Development Centre, which are used at RSU Institute of Dentistry to replace bone defects, significantly increase mineral density and improve biomechanical properties after implantation in osteoporotic bone.

Research structure (design)

Experimental comparative study with placebo surgery and control group.

Study groups: female rabbits with experimentally induced osteoporosis with biphasic calcium granules with and without strontium in the thigh trochanter major area.

Placebo surgery group: animals with experimentally induced osteoporosis without the incorporation of biomaterial granules into the bone.

Control group: healthy animals.

Research places

Laboratory of Biomechanics, Rīga Stradiņš University – determination of biomechanical properties of bone samples.

Rīga Stradiņš University, Institute of Dentistry – determination of mineral density of bone samples by computed tomography.

Rīga Stradiņš University, Department of Pathology – morphological analysis of bone samples.

“Liepudent” dental clinic dental technical laboratory – preparation of bone samples for the study of biomechanical properties.

Ethical aspects

The research has been performed in accordance with the ethical norms that have been established for the performance of experimental research. The research has been issued a permit from the Latvian Food and Veterinary Administration and a permit from the Ethics Committee of Rīga Stradiņš University.

Structure and volume of the Thesis

The dissertation is written in the Latvian language. It has a classical structure and consists of 10 parts: introduction, literature review, material and methods, results, discussion, conclusions, list of used literature, the Author's publications and theses on the research topic, acknowledgments and appendices.

The volume of the dissertation: 106 pages, including 45 images and 1 table. The list of used literature includes 174 sources of scientific articles and 6 books.

1. Material and methods

1.1. Research materials

1.1.1. Animal model

The study used 34 eight-months-old female experimental animals – California and Great Marders rabbits from the rabbit farm “Podziņa”, Ādaži vicinity, Latvia LV-2164, holding No. 1382580, herd No. LV06178330 (RSU Procurement Commission Decision No. 5-2 / 80 (17.02.2015)) with an average weight before the experiment of 3.87 kg (2.9–5.0).

The animals were kept in the Experimental Animal Farm of Rīga Stradiņš University (actual address Kristapa Helmaņa Street 8, Jelgava, LV-3004). The hospital (32.8 m²) and the surgery hall (62.0 m²) were leased from the Latvia University of Life Sciences and Technologies under contract No. 4.4.-9/15/2015. During the study, the requirements were observed in accordance with the Cabinet of Ministers Regulations No. 52 “Regulations on the Protection of Animals Used for Scientific Purposes”. The animals were housed in 100 × 45 × 62 cm cages. Hay and oats were used for animal feed. Tap water was used for the animals. During the experiment, all rabbits had unrestricted access to food and water.

Experimental osteoporosis was induced in 24 rabbits after ovariectomy and subsequent injection of methylprednisolone. One rabbit *Exitus letalis* on day 28 after the start of the methylprednisolone course. There were 10 healthy rabbits in the control group. Biomaterial implantation surgeries and animal euthanasia were performed between February 20, 2016 and May 13, 2016.

Protocol for the induction of experimental osteoporosis

Experimental osteoporosis was based on the principles of the methodology previously described in the scientific literature (*Baofeng et al.*, 2010; *Wanderman et al.*, 2018). Surgical manipulations were performed under general anaesthesia using a 10 % solution of ketamine at a dose of 30 mg / kg body weight, a 2 % solution of xylazine at a dose of 3 mg / kg body weight, and a 0.1 % solution of atropine at a dose of 0.1–0.5 mg / kg body weight. In the first stage of surgery, after preparation of the surgical field, a 3 cm incision was made in the anterior abdominal wall near the navel along the midline (operated by prof. A. Skaĝers, assoc. prof. Ğ. Šalms). The abdominal cavity was opened, the ovaries were found and cut at the base after ligation, and the haemostasis was checked (Figure 1.1.).



Figure 1.1. Bilateral ovariectomy surgery
(photo from doc. I. Šalma's personal archive)

The abdominal wall was closed in two layers with absorbent 4/0 threads. The skin was closed without tension with non-absorbent 4/0 threads, which were removed on the tenth postoperative day. Postoperative pain was controlled with subcutaneous injections of 1 to 3 mg / kg ketoprofen daily for the first three days.

Following wound healing and recovery, a 6-week course of methylprednisolone intramuscularly at 1 mg / kg / day was initiated after

14 days. During the course of methylprednisolone, the animals received the hepatoprotector Hepatiale Forte 170 mg daily (phospholipids 85 mg, L-ornithine L-aspartate 85 mg).

Drug selection and dosages are based on the associate Professor Aija Ilgaža's recommendations from LLU Veterinary Medicine Preclinical Institute of Veterinary Medicine and data from *James W. Carpenter "Exotic Animal Formulary"* and *Ian Ramsey "BSAVA Small Animal Formulary"*. The use of veterinary drugs for rabbits was performed by a specialist veterinarian Jekaterina Petļa, who also provided welfare care for the experimental animals in the postoperative period.

Bone defect formation in placebo and with biomaterial implantation samples

In the second stage of surgery, an approximately 2 cm long incision was made in the right *trochanter major* region of the thigh under general anaesthesia, the soft tissues and periosteum were detached (Figure 1.2.).



Figure 1.2. *Trochanter major* area of the right thigh
(photo from doc. I. Šalma's personal archive)

A circular defect in the large region of the thigh was created with a steppe drill 5 mm in diameter and 4 mm deep (Figure 1.3).



Figure 1.3. **5 × 4 bone defect in the *trochanter major* area of the thigh**
(photo from doc. I. Šalma's personal archive)

The 23 experimental animals were divided into three groups: Group 1 (8 rabbits) – defects were filled with HAP/TCP at 70/30 granules, Group 2 (8 rabbits) – defects were filled with HAP/TCP at 70/30 granules with 5 % strontium, in placebo surgery group 3 (7 rabbits) – defects were made, but no material was inserted (Figure 1.4).



Figure 1.4. **Biomaterial implantation in the *trochanter major* area of the thigh**
(photo from doc. I. Šalma's personal archive)

The periosteum above the bone defect was closed with absorbent 4/0 sutures; the skin was closed without tension with non-absorbent 4/0 threads, which were removed on the tenth postoperative day (Figure 1.5).



Figure 1.5. Periost closure with absorbent threads
(photo from doc. I. Šalma's personal archive)

Animals were observed for water, food intake, weight changes, suture, wound condition, animal behaviour, appearance. No loss of physical activity or appetite was observed during the experiment.

Animal euthanasia and bone sample obtaining

Animals were euthanised three months after the onset of a bone defect under general anaesthesia with intrapulmonary administration of T-61 at a dose of 1.0 ml / kg body weight. The minimum number of samples to obtain statistically reliable results was more than 6 (*Yamada, 1970; Evans, 1971; Charan et al., 2013*).

To demonstrate osteoporosis by morphological examination, 10 samples from the premolar region of mandible from healthy rabbits and 23 samples from the premolar region of mandible from rabbits with experimental osteoporosis were taken. To determine the local effects of biomaterials, 23 samples of the right (operated) thigh from rabbits with experimental osteoporosis were used. 23 samples of the left (non-operated) thigh and

23 samples from angle of mandible from rabbits with experimental osteoporosis were used to determine systemic biomaterial influence with X-ray examination methods (Figure 1.6).

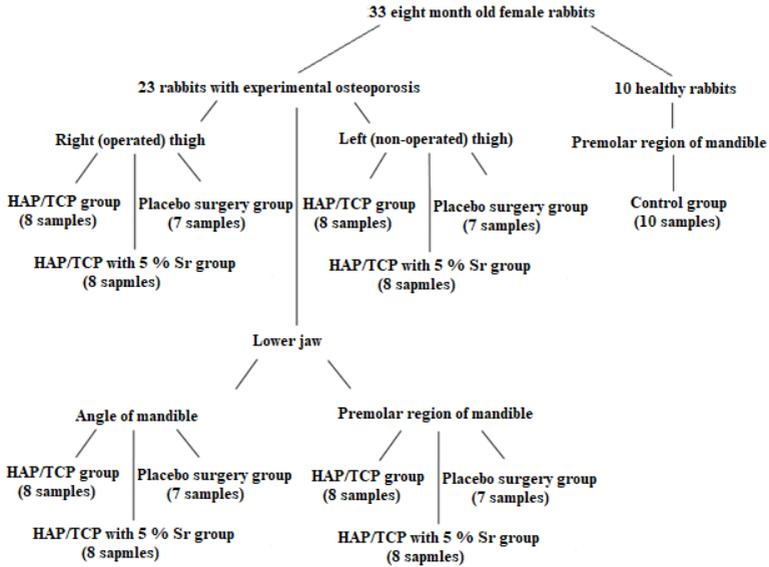


Figure 1.6. Distribution scheme of the rabbit bone samples

1.1.2. Biomaterial preparation

Non-commercial biomaterials produced by Riga Technical University Rudolfs Cimdins Biomaterials Innovation and Development Centre were used for the research. The synthesis of calcium phosphate materials and the preparation of granules were carried out as previously described in the scientific literature (*Grybauskas et al.*, 2016; *Zarins et al.*, 2017). Prior to calcium phosphate synthesis, CaO (Fluka) and SrO (Sigma-Aldrich) are heat treated in a high temperature oven at 1100 °C for 1 hour for activation purposes. From the obtained calcium oxide and strontium oxide, 0.3M Ca (OH)₂ or Sr (OH)₂/Ca (OH)₂ suspensions in deionised water are prepared. The synthesis of calcium

phosphates was performed in a reactor by adding $2\text{MH}_3\text{PO}_4$ to the hydroxide suspensions during their mixing in such an amount that the HAP/TCP phase ratio of the obtained product after high temperature treatment would be 70/30. After synthesis, the precipitate is filtered and granulated using a manual extrusion method. The resulting granules are dried and fired at $1150\text{ }^\circ\text{C}$ for 2 hours. The heat-treated pellets are divided into fractions by sieving with a vibrating sieve machine using sieves with a mesh size of $1000\text{ }\mu\text{m}$ and $500\text{ }\mu\text{m}$, thus obtaining the required granule fraction (from 0.5 to 1 mm). After sieving, the granules are washed several times in ethanol to remove fines and dried at $70\text{ }^\circ\text{C}$.

For the *in vivo* experimental study, the biomaterial was tested with physico-chemical and cell cultures *in vitro* after preparation, selecting the least toxic, biocompatible and most effective particles in the direction of osteogenic bioactivity.

Characteristics of biomaterial granules can be found in table 1.1.

Table 1.1.

Characteristics of biomaterial granules.

Biomaterial	Surface area, m^2/g	Bulk density, g/cm^3	HAP/TCP content, %	Sr content, %
HAP/TCP (70/30)	1.262 ± 0.014	0.98 ± 0.01	73/27	0.017 ± 0.002
HAP/TCP with Sr (70/30)	1.124 ± 0.0147	1.05 ± 0.02	71/29	6.7 ± 0.7

Designation: HAP – hydroxyapatite, TCP – tricalcium phosphate, Sr – strontium

1.2. Research methods

1.2.1. Biomechanical research method

Within the framework of the study, a three-point bending test was performed to determine the biomechanical parameters of bone tissue (*Prodingier et al.*, 2018), (Figure 1.7).

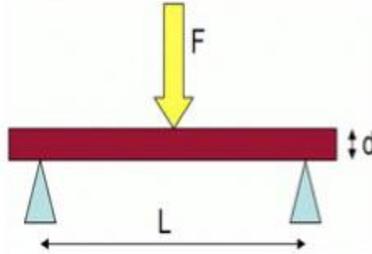


Figure 1.7. **Three-point bending test scheme of the sample:**
F – load, L – span, d – sample thickness

During bending, the ultimate (maximum) stress (σ^*) and ultimate (maximum) strain (ε^*) were determined for each sample. The computer programme presented the measurement results in tabular form and graphically as a stress-strain curve. The hardness of the specimens was expressed as the tangential flexure modulus on a linear part of the stress-strain curve as the tangent angle α ($\text{tg } \alpha$) formed between the coordinate axis and the tangent curve in its linear part (Figure 1.8).

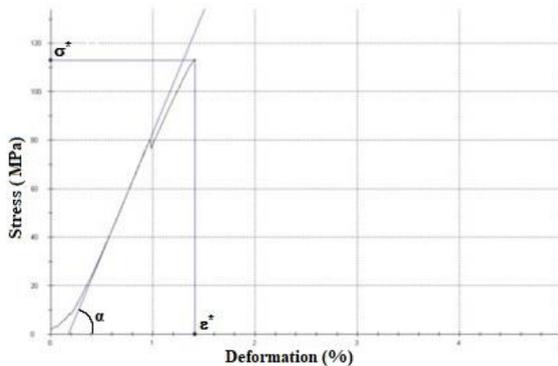


Figure 1.8. Typical experimental stress-strain relationship and main mechanical parameters to be determined: σ^* – ultimate stress, ε^* – ultimate strain, tg α – flexure modulus

The *ultimate stress* was calculated according to the formula:

$$\sigma = 3FL / (2bd^2),$$

where F – load, L – span, b – sample width, d – sample thickness.

The ultimate stress was measured in pascal (Pa).

The *ultimate strain* was calculated according to the formula:

$$\varepsilon = 6df / L^2,$$

where d – thickness of the sample, f – inclination of the sample and L – span.

The ultimate strain was determined in percentages (%).

The *flexure modulus* was calculated according to the formula:

$$E = FL^3 / (4fbd^3)$$

where F – load, L – span, f – sample inclination, b – sample width, d – sample thickness.

The flexure modulus was measured in pascal (Pa).

The flexure modulus was calculated in the linear part of the stress-strain curve during the strain of the sample ($\varepsilon = 0.5 \%$).

Rectangular specimens were used to study the biomechanical properties of bone tissue by three-point bending test. Rectangular bone blocks were obtained using a *Surgic Pro* physiodispenser (NSK, Japan), a straight tip, and a 5 mm diameter diamond disc drill (Figure 1.9).



Figure 1.9. *Surgic pro* physiodispenser for obtaining rectangular bone samples
(photo from the Author's personal archive)

Rectangular bone samples were obtained from the diaphysis of the operated thigh, the diaphysis of the non-operated thigh, and the angular region of the mandible (Figure 1.10).

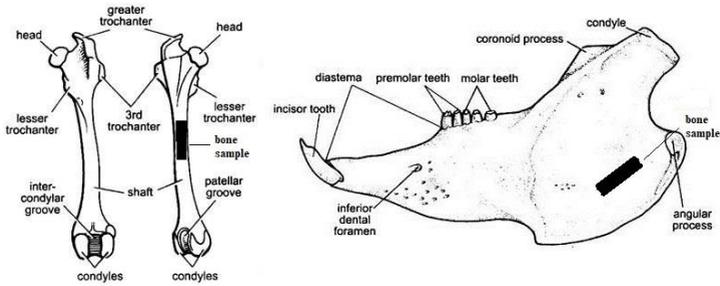


Figure 1.10. Schematic drawing of rabbit thigh and mandible bone – cut-out of bone samples marked with a black rectangle

(Endoskeleton of Rabbit (With Diagram). NotesOnZoology.com, Fig. 29.11 un 29.19. (<http://www.notesonzology.com/rabbit/endoskeleton/endoskeleton-of-rabbit-with-diagram-vertebrates-chordata-zoology/7690>))

All samples were aligned to one size using a specially made sample calibration device and an abrasive grinding wheel: thigh bone sample width 3.2 ± 0.02 mm, thickness 0.61 ± 0.02 mm; width of mandibular bone samples 3.2 ± 0.02 mm, thickness 0.32 ± 0.02 mm. A special device for calibrating samples was manufactured and calibrated in the Liepādent Dental Technical Laboratory. Samples that did not meet certain sizes were excluded from the study (Figure 1.11).



Figure 1.11. Specially made device for calibration of samples and abrasive grinding wheel for obtaining bone samples
(photo from the Author's personal archive)

Width and thickness were measured with a cathetermetre *KM-6* (Lomo, Russia), measurement accuracy ± 0.01 mm (Figure 1.12).



Figure 1.12. **KM-6 catheter, accuracy of measurement ± 0.01 mm**
(photograph from the Author's personal archive)

Bone samples were measured at a distance of 550 mm from the catheter (Figure 1.13).



Figure 1.13. **Measurement of the width and thickness of the sample**
(photo from the Author's personal archive)

The bending of the samples was performed on a stand *Zwick/Roell* (BT1-FR2.5TN.D14, Germany) equipped with a 2.50 ± 0.1 kN force strain gauge (Figure 1.14).



Figure 1.14. **Zwick/Roell bending test stand**
(photo from the Author's personal archive)

Bone samples were placed 10 mm between the supports (Figure 1.15).

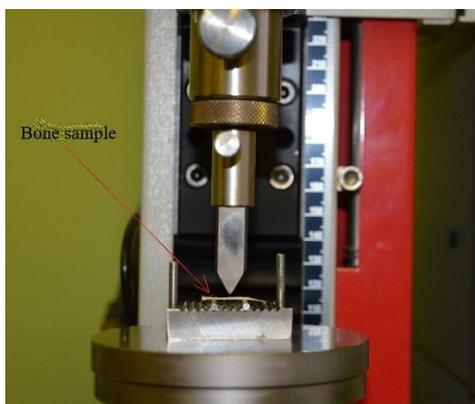


Figure 1.15. **Research of a sample for a three-point bending test**
(photo from the Author's personal archive)

The stand was used together with the computer programme *testXpert 2.5* (Germany), which is intended for control of the test device and data processing. The samples were deformed at a rate of 1 mm/min until the sample collapsed.

The data obtained on the load bench were processed with *testXpert 2.5* programme.

At each point of the stress-strain curve after the bench pressure nozzle displacement programme, the inclination of the specimen is automatically calculated. According to the formulas that were entered into the programme, the ultimate stress, ultimate strain and flexure modulus were automatically calculated.

1.2.2. Radiological research method

Operated and non-operated thigh cortical bone density was measured by cone beam computed tomography using *iCAT Next Generation* (Kavo, Germany) (Figure 1.16).



Figure 1.16. *iCAT Next Generation* cone beam computed tomography (photo from the Author's personal archive)

The equipment was used with a standard operating protocol (voltage 120 KV, current 38 mA, field of view (FOV) 17 cm, resolution 0.4 voxels (three-dimensional image volume unit)). The obtained test data were processed and analysed using hardware-specific software *iCAT eXamVision* (Kavo, Germany). Image voxel blackening level values were determined for the samples. The

values displayed in *Hounsfield units* (HU) were converted to 1:1 radiological voxel blackening level value units (*Do-Gyoon Kim*, 2014). The blackout level value of the image is determined in a field of 4 mm² (Figure 1.17).

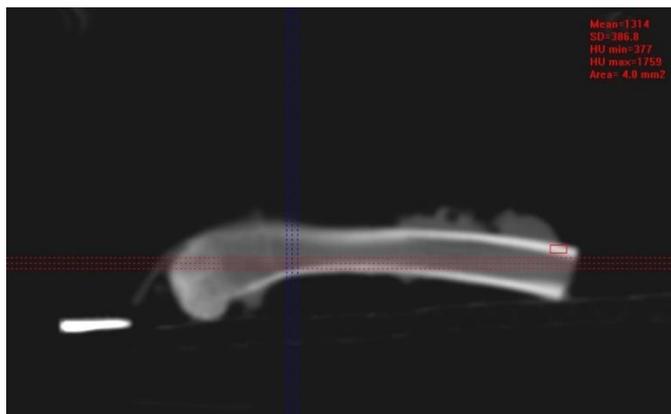


Figure 1.17. Cone beam computed tomography image to determine the value of the blackening density of the radiological image in the cortical bone of the thigh body – in the sagittal plane. Red lines – coronary plane, blue lines – axial plane. The field under investigation is marked with a red rectangle. In the upper right corner, the named parameters can be seen: *Mean* – average bone mineral density in the examined field; *SD* – standard deviation; *HU min* – minimum bone mineral density in the examined field; *HU max* – maximum bone mineral density in the examined field; *Area* – size of the field to be examined

1.2.3. Morphological research method

The study used the classical tissue preparation method for 33 bone samples from the premolar region of the mandible. A routine screening method with hematoxylin/eosin was used for bone morphological examination (*Kiernan*, 2015).

After removal of the bone blocks, the tissues were placed in a fixative solution of 10 % formalin for two weeks. After fixation, the bones were decalcified with a solution of *Osteodec* (Bio-medica, Italy), then the tissues were

dehydrated with ethyl alcohol and degreased in a xylene solution. This was followed by tissue loading into paraffin cassettes to form paraffin blocks, from which 3–5 μm thick sections were prepared and applied to slides for further processing and hematoxylin/eosin staining.

The trabecular bone area was measured using *Image Pro Plus 7* software (Media Cybernetics, USA). Three identical fields of view (0.975 mm²) were randomly selected in all bone samples.

1.3. Statistical data processing methods

Statistical processing, calculation and graphical representation of all data were performed with special software *GraphPad PRISM v.6.0e* (GraphPad Software Inc., San Diego, California, USA). The homogeneity of the dispersion was checked by *Brown-Forsythe* and *Bartlett* tests. In the case of uneven distribution, the mean values of the numerical variables of different groups were compared using the non-parametric ANOVA test or *Kruskal-Wallis* test and then using the *Benjamini*, *Krieger* and *Yekutieli* method as a *post-hoc* procedure.

All results were expressed as medians (Md) with interquartile range (IQR). The two values were considered statistically significantly different if the level of confidence between them was greater than 95 % ($p < 0.05$).

2. Results

2.1. Results of biomechanical research and statistical data processing in thigh bone samples

All bone samples used to determine biomechanical parameters of the thigh were divided into three groups:

- 1) Placebo surgery group: defect was made in the right thigh in the *trochanter major* area, but no material is inserted;
- 2) HAP/TCP group: defect in the right thigh *trochanter major* region were filled with HAP/TCP in a ratio of 70/30 granules;
- 3) HAP/TCP/5% Sr group: defect in the right thigh *trochanter major* region were filled with HAP / TCP in 70/30 granules with 5% strontium.

2.1.1. Results of ultimate strain determination and statistical data processing

The ultimate strain of the *operated thigh* placebo surgery group samples was 1.43 (1.57–1.20) %, which is statistically significant more than the HAP/TCP group samples 1.16 (1.18–1.13) %, $p = 0.002$, and the HAP/TCP/5 % Sr group samples. 1.04 (1.14–0.86) %, $p = 0.005$. Statistically significant differences in the ultimate strain of the samples between HAP/TCP and HAP/TCP/5 % Sr group samples were found: the ultimate strain of bone samples of HAP/TCP group is greater than the ultimate strain of bone samples of HAP/TCP/5 % Sr group, $p = 0.02$ (Fig. 2.1.).

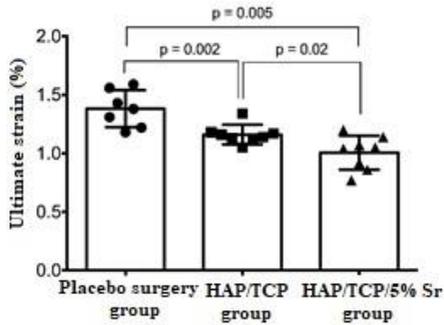


Fig. 2.1. Ultimate strain of the operated thigh samples for different groups

The ultimate strain of the *non-operated thigh* placebo group was 1.27 (1.42–1.17) % , which is statistically significantly more than the HAP/TCP group 1.06 (1.15–0.93) % , $p = 0.012$, and the HAP/TCP/5% Sr group 1.02 (1.14–0.9) % , $p = 0.003$. No statistically significant differences in the ultimate strain of the samples between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p = 0.64$ (Figure 2.2.).

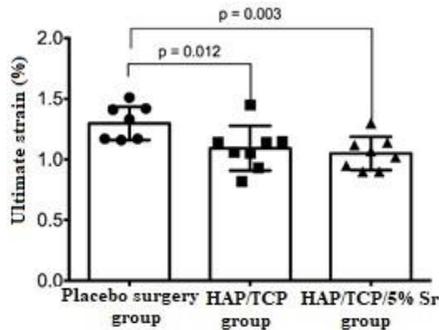


Figure 2.2. Ultimate strain of the non-operated thigh samples for different groups

2.1.2. Results of ultimate stress determination and statistical data processing

No differences in reliability of the ultimate stress statistics of the *operated thigh* samples were observed between all three samples over time ($p > 0.05$): the ultimate stress values for the placebo surgery group samples were 176.7 MPa (216–154), for the HAP/TCP group samples 194.6 MPa (207.2–169.9) and HAP/TCP/5% Sr group samples 193.7 MPa (208.1–176.1) (Figure 2.3).

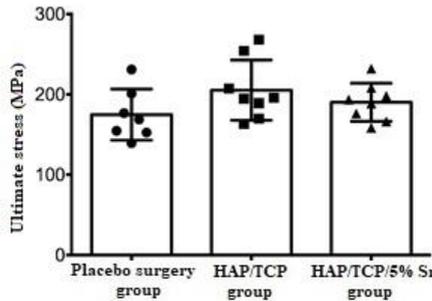


Figure 2.3. **The ultimate stress of the operated thigh samples for different groups**

Samples from the *non-operated thigh* placebo surgery group had 155.5 MPa (174.7–140.8), which is statistically significantly less than the HAP/TCP group 209.2 MPa (234.2–164.4), $p = 0.017$, and HAP/TCP/5% Sr group samples. 177.7 MPa (200.3–165.5), $p = 0.026$. No statistically significant differences in the ultimate stress of the samples between HAP/TCP and HAP/ TCP/5% Sr group samples were found, $p = 0.46$ (Figure 2.4).

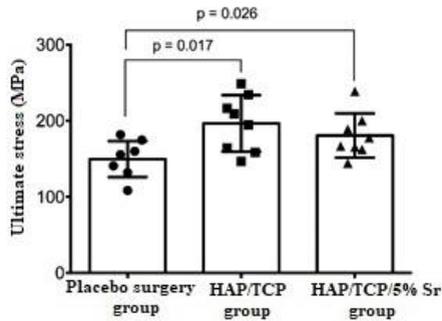


Figure 2.4. **Ultimate stress of the non-operated thigh samples for different groups**

2.1.3. Results of flexure modulus determination and statistical data processing

The flexure modulus describes the stiffness of the material: the higher the flexure modulus, the stiffer the material. The results of the study showed that the flexure modulus of the *operated thigh* placebo surgery group samples was 16.31 GPa (18.66–12.15), which is statistically significantly less than the HAP/TCP group samples 19.87 GPa (21.10–17.94), $p = 0.01$, and HAP/TCP/5% for Sr group samples 21.88 GPa (27.53–21.09), $p = 0.005$. Statistically significant differences were found in the flexure modulus of the samples between HAP/TCP and HAP/TCP/5% Sr group samples: the flexure modulus of bone samples of HAP/TCP group is lower than the flexure modulus of bone samples of HAP/TCP/5% Sr group, $p = 0.02$ (Figure 2.5).

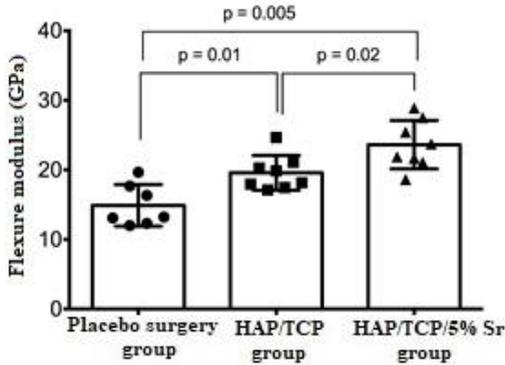


Figure 2.5. Flexure modulus of the operated thigh samples for different groups

The flexure modulus of the *non-operated thigh* placebo surgery group samples was 14.91 GPa (17.36–13.6), which is statistically significantly less than the HAP/TCP group samples 21.86 GPa (25.81–19.6), $p = 0.004$, and the HAP/TCP/5% Sr group samples 22.11 GPa (23.41–20.29), $p = 0.006$. No statistically significant differences in the bending modulus of the samples between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p = 0.87$ (Figure 2.6).

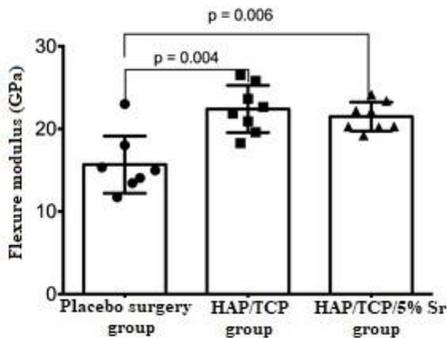


Figure 2.6. Flexure modulus of non-operated thigh samples for different groups

2.2. Results of biomechanical research and statistical data processing in mandible bone samples

All bone samples used to determine biomechanical parameters of the mandible bone were divided into three groups:

- 1) Placebo surgery group: defect was made in the right thigh in the *trochanter major* region, but no material was inserted;
- 2) HAP/TCP group: defect in the right thigh *trochanter major* region were filled with HAP/TCP in a ratio of 70/30 granules;
- 3) HAP/TCP/5% Sr group: defect in the right thigh *trochanter major* region were filled with HAP/TCP in a ratio of 70/30 granules with 5 % strontium.

2.2.1. Results of ultimate strain determination and statistical data processing

The ultimate strain of the placebo surgery group samples was 2.04 % (2.37–1.76), which is statistically more reliable than the HAP/TCP group samples 1.46 % (1.49–1.27), $p = 0.0003$, and the HAP/TCP/5% Sr group samples 1.37 % (1.94–1.03), $p = 0.02$. No statistically significant differences in the ultimate strain of the samples between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p > 0.99$ (Figure 2.7.).

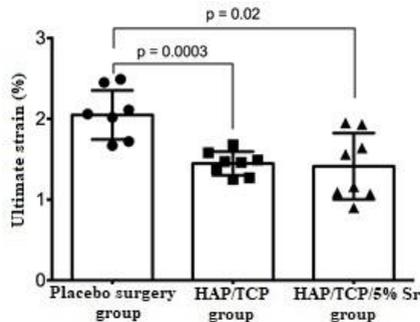


Figure 2.7. Ultimate strain of mandibular bone samples for different groups

2.2.2. Results of ultimate stress determination and statistical data processing

The ultimate stress for the placebo surgery group samples was 89.36 MPa (108.6–77.44), which is statistically significantly less than for the HAP/TCP group samples 183 MPa (202.7–155.2), $p = 0.0003$, and for the HAP/TCP/5% Sr group samples 241.9 MPa (250.9–234.9), $p = 0.0007$. Statistically significant differences in the ultimate stress of the samples between the HAP/TCP and HAP/TCP/5% Sr groups were found: the ultimate stress of the bone samples of the HAP/TCP group is less than the ultimate stress of the bone samples of the HAP/TCP/5% Sr group, $p = 0.022$ (Figure 2.8.).

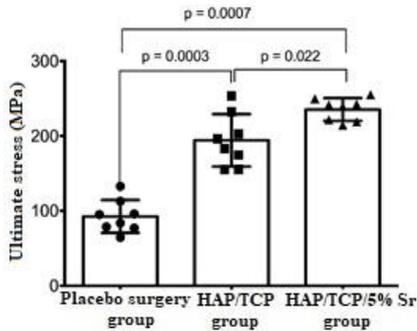


Figure 2.8. Ultimate stress of mandibular bone samples for different groups

2.2.3. Results of flexure modulus determination and statistical data processing

The flexure modulus of the placebo group samples was 5.25 GPa (7.23–4.35), which is statistically significantly less than the HAP/TCP group samples 12.44 GPa (17.71–11.55), $p = 0.0003$, and the HAP/TCP/5% Sr group samples 15.83 GPa (20.8–14.67), $p = 0.0007$. No statistically significant differences in

the flexure modulus of the samples between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p = 0.29$ (Figure 2.9).

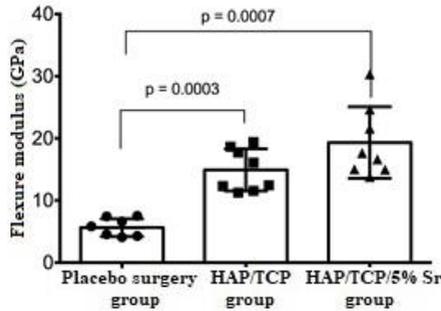


Figure 2.9. Flexure modulus of mandibular bone samples for different groups

2.3. Results of radiological research and statistical data processing in thigh bone samples

All bone samples used to determine thigh mineral density were divided into three groups:

- 1) Placebo surgery group: defect was made in the right thigh *trochanter major* region, but no material was inserted;
- 2) HAP/TCP group: defect in the right thigh *trochanter major* region were filled with HAP / TCP in a ratio of 70/30 granules;
- 3) HAP/TCP/5% Sr group: defect in the right thigh *trochanter major* region were filled with HAP / TCP in 70/30 granules with 5 % strontium.

Samples of the *operated thigh* placebo surgery group had a cortical bone density of 923 HU (1056–843.5), which is statistically significantly less than samples of the HAP/TCP group 1302 HU (1350–1209), $p = 0.016$, and HAP/TCP/5% Sr for samples 1344 HU (1459–1156), $p = 0.042$. No statistically

significant differences in cortical bone density between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p = 0.761$ (Figure 2.10.).

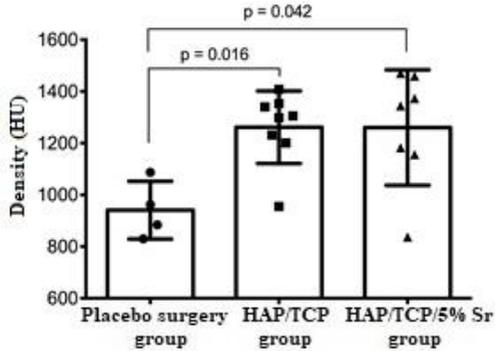


Figure 2.10. **Cortical bone density of the operated thigh samples for different groups**

Samples of the *non-operated thigh* placebo surgery group had a cortical bone density of 935.5 HU (1053–876), which is statistically significantly less than samples of the HAP/TCP group 1212 HU (1360–1138), $p = 0.016$, and HAP/TCP/5% of the Sr group. for samples 1261 HU (1315–1090), $p = 0.012$. No statistically significant differences in cortical bone density between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p > 0.99$ (Figure 2.11.).

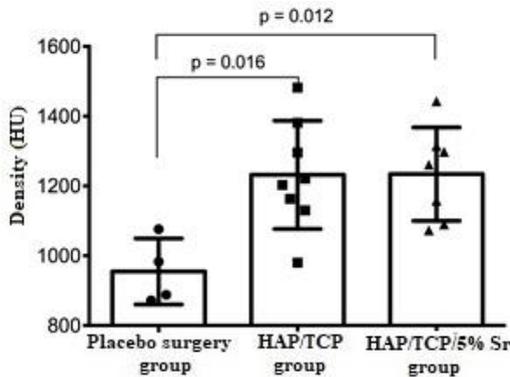


Figure 2.11. **Cortical bone density of non-operated thigh samples for different groups**

2.4. Results of radiological examination and statistical data processing in mandibular bone samples

All bone samples used to determine mandibular bone mineral density were divided into three groups:

- 1) Placebo surgery group: defect was made in the right thigh *trochanter major* region, but no material was inserted;
- 2) HAP/TCP group: defect in the right thigh *trochanter major* region were filled with HAP/TCP in a ratio of 70/30 granules;
- 3) HAP/TCP/5% Sr group: defect in the right thigh *trochanter major* region were filled with HAP/TCP in 70/30 granules with 5 % strontium.

Samples in the placebo surgery group had a cortical bone density of 709.5 HU (807–645), which is statistically significantly less than in the HAP/TCP group 871 HU (1047–829), $p = 0.024$, and in the HAP/TCP/5% Sr group 916 HU (962–876), $p = 0.006$. No statistically significant differences in cortical bone density between samples between HAP/TCP and HAP/TCP/5% Sr group samples were found, $p = 0.522$ (Figure 2.12).

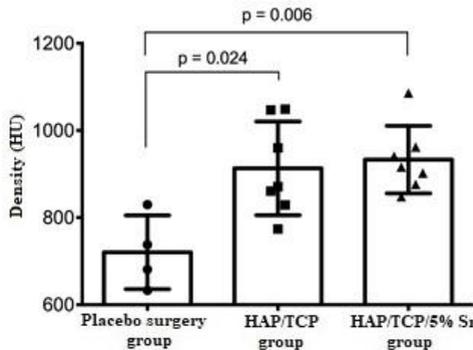


Figure 2.12. Cortical bone density of mandibular bone samples for different groups

2.5. Comparison of biomechanical parameters and mineral bone density of operated and non-operated thighs after implantation of different biomaterials

All bone samples used to compare biomechanical parameters and density of operated and non-operated femurs were divided into four groups:

- 1) HAP/TCP LT group: operated thigh bone samples – defect in the right thigh *trochanter major* region were filled with HAP/TCP in a ratio of 70/30 granules;
- 2) HAP/TCP RT group: non-operated thigh samples – defect in the right thigh *trochanter major* region were filled with HAP/TCP in the ratio of 70/30 granules;
- 3) HAP/TCP/5% Sr LT group: operated thigh bone samples – in the right thigh *trochanter major* region, defects were filled with HAP/TCP in a ratio of 70/30 granules with 5 % strontium;
- 4) HAP/TCP/5% Sr RT group: non-operated thigh bones samples – in the right thigh *trochanter major* region, defects were filled with HAP/TCP in a ratio of 70/30 granules with 5 % strontium.

Comparing the ultimate strain, ultimate stress, flexure modulus and mineral bone density of operated and non-operated thigh samples, no statistically significant differences were found ($p > 0.05$), (Figures 2.13.–2.16.).

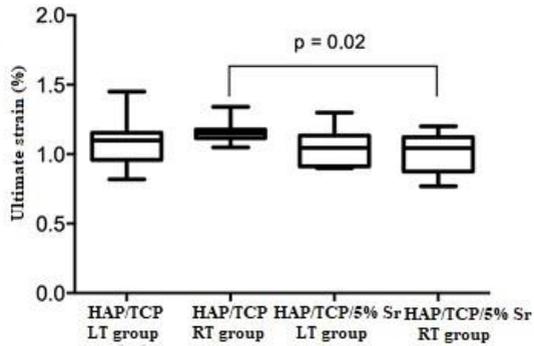


Figure 2.13. Comparison of ultimate strain of operated and non-operated thigh samples after implantation of different biomaterials

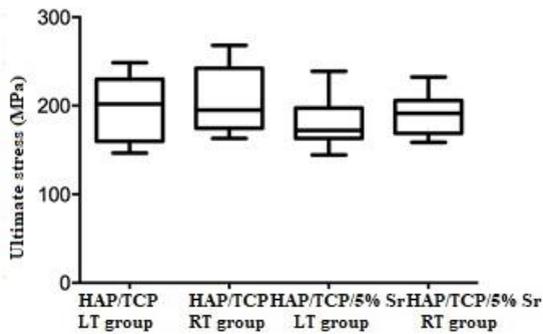


Figure 2.14. Comparison of the ultimate stress of operated and non-operated thigh samples after implantation of different biomaterials

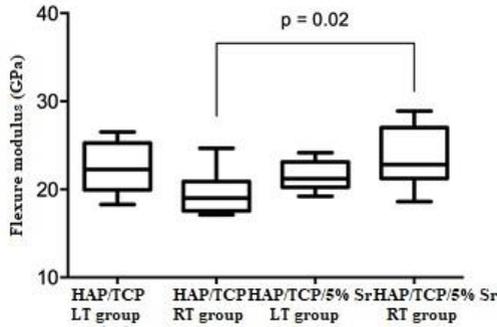


Figure 2.15. Comparison of the flexure modulus of operated and non-operated thigh samples after implantation of different biomaterials

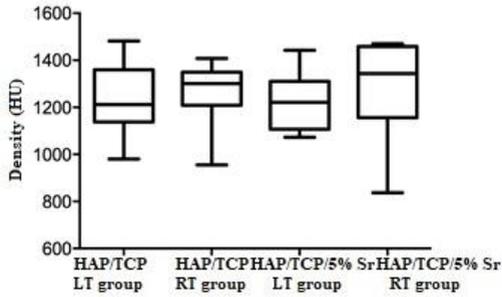


Figure 2.16. Comparison of mineral bone density of operated and non-operated thigh samples after implantation of different biomaterials

2.6. Results of morphological examination and statistical data processing in mandibular bone samples

All bone samples used to determine the trabecular bone area of the mandible were divided into four groups:

- 1) Control group: healthy rabbits;
- 2) Placebo surgery group: defect was made in the right thigh *trochanter major* area, but no material was inserted;

- 3) HAP/TCP group: defect in the right thigh *trochanter major* region was filled with HAP/TCP in a ratio of 70/30 granules;
- 4) HAP/TCP/5% Sr group: defect in the right thigh *trochanter major* region was filled with HAP/TCP in 70/30 granules with 5 % strontium.

The trabecular bone area in the control group samples was larger compared to the osteoporotic groups (placebo surgery group, HAP/TCP group, HAP/TCP5% Sr group) samples. The trabecular bone area in the control group was 0.20 mm² (0.176–0.233 mm²), which is statistically significantly larger ($p < 0.0001$) than in the HAP/TCP group (0.127 mm²; 0.118–0.149 mm²), in the HAP/TCP/5% Sr group (0.136 mm²; 0.108–0.166 mm²) and placebo surgery group (0.135 mm²; 0.126–0.164 mm²). No statistically significant differences were found between the HAP/TCP, HAP/TCP/5% Sr and placebo surgery groups, $p > 0.05$ (Figure 34).

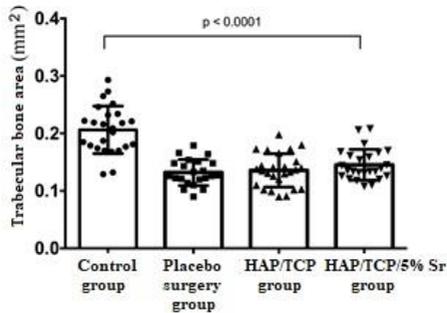


Figure 2.17. Trabecular bone area for different groups

3. Discussion

The work of the study was implemented in the State research programme No. 2014.10-4/VPP-3/21 “Multifunctional materials and composites, photonics and nanotechnologies”, project No. 4 “Nanomaterials and nanotechnologies for medical applications” and is part of the large project aimed at creating and researching new, applicable and competitive biomaterials – nanostructured composites for strengthening and replacement of osteoporotic bone. As living standards rise, each country faces an aging population associated with people whose quality of life depends on musculoskeletal disorders, the second most common cause of disability globally (*Sözen et al.*, 2017). According to the data of the European Union, approximately 33 % of the population in Latvia (at about 700,000 people) suffer from musculoskeletal diseases, such as osteoporosis, rheumatoid arthritis, osteoarthritis, spondylosis, gout and more than 200 other chronic diseases and a person quickly becomes incapacitated for work.

In addition to the general drug treatment of osteoporosis, studies have recently been conducted on osteoporotic bone augmentation for fracture prevention in the most common threatening fracture sites (*Cosman et al.*, 2014; *Iaquinta et al.*, 2019). *Iaquinta et al.* review considered various aspects of tissue engineering that were used in bone engineering. The most studied bone tissue replacement materials are implanted, modifying them in the direction of new structures and technologies, which is also the focus of this experimental study. The current study, which was implemented within the framework of the VPP project, also aimed to investigate new multifunctional materials and methods for strengthening bone volume and mechanical properties, bone remineralisation and bone reosification – turning bone remodelling from atrophy to bone remodelling – osteoporotic bone treatment with biphasic calcium phosphate bioceramic materials with strontium ion administration studied and used in the clinic.

In vivo observations play an important role in the development of biocompatible biomaterials, because only after a positive opinion on the biocompatibility of the material, the material can be forwarded for further clinical research. Prior to *in vivo* studies, there is a rigorous selection of the most suitable biomaterials for *in vitro* studies using cell cultures. Various methods are used to induce osteoporosis in animals, among which bilateral ovariectomy of female animals is the most common. Other methods commonly used to induce osteoporosis include immobilisation, dietary change, and glucocorticoid administration (Calciolari *et al.*, 2017). Similar methods for inducing osteoporosis have been used in the study.

Several animal models have been used to mimic postmenopausal osteoporosis, mainly rodents (Kalu, 1991; Turner, 2001). Rats and mice are commonly used for low cost, ease of holding and transport, but they do not reach full skeletal maturity, the bones do not have a Haversian duct system, there is little remodelling in the cortical bone. These conditions limit the use of rats and mice in osteoporosis studies, especially in drugs that affect bone mineral density, which require an assessment of the dynamics of cortical and trabecular bone remodeling. Wanderman *et al.* concluded that the use of smaller animals complicates implant surgery in technical terms, as well as it is difficult to evaluate the efficacy of osteoporosis drugs due to the peculiarities of bone structure (Wanderman *et al.*, 2018).

In contrast, data from the Kimmel study suggest that the use of larger animals (for example dogs, sheep, monkeys) in experimental surgery would bring them closer to humans in terms of technology, but is not possible for technical reasons and within the allocated funding (Kimmel, 2001). Castaneda *et al.* suggested that the use of rabbits in biomaterial studies is recommended because they have active bone remodelling. The morphological changes observed in rabbit and human tissues are similar and comparable. Rabbits are a

good model for experimental osteoporosis studies because they reach skeletal maturity at 7–8 months and have significant remodelling in cortical bone (Castaneda et al., 2008). Gathering data from the scientific literature, rabbits are used as an animal model in the current experimental study.

Analysis of the literature did not reveal any studies comparing biomechanical parameters or mineral density of bone tissue adjacent to the site of biometric implantation and distantly from the animal model of osteoporosis. In addition, bone samples from two or three groups of animals are often analysed. The current study can be considered unique in that 102 bone samples from 13 different groups of animals were analysed, comparing bone samples after implantation of biphasic calcium phosphate bioceramics with or without strontium with placebo surgery and control bone samples from rabbits with experimental osteoporosis. Biomechanical parameters (ultimate strain, ultimate stress and flexure modulus) and mineral density of osteoporotic bone samples were evaluated. Bone samples were taken from the operated thigh body as close as possible to the implantation site to determine local biometric recommendation, as well as from the non-operated thigh body and mandibular angular area to determine systemic biomaterial exposure.

To demonstrate osteoporosis, the trabecular bone area was determined in the premolar region of the mandibular bone in rabbits with experimental osteoporosis and healthy animals. Statistically significant differences were observed in healthy and osteoporotic rabbits: the trabecular bone area in healthy rabbits was 0.20 mm^2 , while in osteoporotic rabbits it ranged from 0.127 mm^2 to 0.136 mm^2 , which is statistically significantly less compared to the trabecular bone area of healthy rabbits. In this study, no statistically significant differences in trabecular bone area were found between the groups of osteoporotic animals 3 months after biomaterial implantation and placebo surgery.

Similar results are reported by *Baier et al.* in a study comparing calcium phosphate cement with strontium-containing cationic phosphate cement, considering their local and systemic effects on bone in an experimental animal model of osteoporosis. In his study, the Author found similar bone volumes at 1 and 3 months after implantation of calcium phosphate-containing biomaterial with or without the presence of strontium. However, when analysing rat bone samples at 6 months, the highest amount of trabecular bone was in the group of strontium-containing biomaterials (*Baier et al.*, 2013). This was also confirmed by *Ni et al.* study, in which strontium-containing hydroxyapatite cement was used in primary hip replacement in a rabbit model. In the author's work, it was found that after 6 months in the presence of strontium a larger amount of bone is formed compared to the group of rabbits, where PMMA cement was used as a bone replacement biomaterial (*Ni et al.*, 2006). The data obtained in the current study and other authors show that the amount of newly formed bones as a result of strontium ions depends on the time period after implantation.

Among the various available bone replacement biomaterials, only autologous bone grafts have osteoconductive, osteoinductive, and osteogenic properties (*Sakkas et al.*, 2017). However, this biomaterial has unpredictable absorption properties, is in limited quantities, and there is a risk of infection (*Nkenke et al.*, 2014; *Jensen et al.*, 2016). Therefore, great attention is paid to the research of bioceramic materials (hydroxyapatite), which must have biocompatible, osteoconductive and biodegradable properties. In *Schlickewei et al.* study defects in the tibia of healthy rabbits were filled with HAP bioceramic granules. New bone formation was observed after 12 weeks. Not only was a bone graft implanted in the animal bone, but new bone also formed on its surfaces (*Schlickewei et al.*, 2015). The results of the present study show an improvement in bone biomechanical parameters and an increase in mineral density in groups of experimental animals after biomaterial implantation. In turn, a similar

trabecular bone area for all groups of osteoporotic animals indicates a constant bone volume.

Biphasic calcium phosphate biomaterials can be used as bone replacement materials to strengthen osteoporotic bone and improve bone healing (*Salma et al.*, 2013). In *Sulaiman et al.* study, it was found that 80 % tricalcium phosphate and 20 % hydroxyapatite combination provide mechanical strength and account for 60–70 % of bone tissue. Tricalcium phosphate serves as a rich source of calcium and phosphorus that can be easily assimilated and absorbed (*Sulaiman et al.*, 2013). It is highly biocompatible, creating a resorbable blocking network at the site of the defect to promote healing and ensure the initial release of calcium and orthophosphate ions into the blood vessels, thus ensuring new bone formation (*Sulaiman et al.*, 2013; *Salma et al.*, 2015).

Ishakawa et al. study compared three commercial bone substitutes with different compositions: hydroxyapatite (HAP, Neobone®), carbonate apatite (CO3AP, Cytrans®) and tricalcium phosphate (TCP, Cerasorb®). The results of the study showed that new bone formation and resorption of bone substitute depend on the type of biomaterial. In HAP, 4 weeks after implantation, new bone formation in the area around the existing bone was limited and the amount of new bone was very small. At week 12, the number of bones was about the same for a year. Unlike HAP, CO3AP produced much more bone at both 4 and 12 weeks. Most CO3AP granules were partially replaced by newly formed bone at week 12. Interesting data were in the case of TCP, when no bone formation was observed at week 4 – the material remained at the bone defect, but a large amount of bone tissue is formed 12 weeks after biomaterial implantation and most of the material was absorbed during this time (*Ishakawa et al.*, 2010). The results of the authors of this work justify the choice of biomaterials used in the current study: HAP in bioceramics was used to provide mechanical strength to the newly formed bone, while TCP ensures the formation of new bone.

The largest amounts of calcium phosphate biomaterials are not homogeneous and contain trace elements that play an important role in growth and bone regeneration (*Ehret et al., 2017*). Strontium is biologically similar to calcium and therefore accumulates in bones. In the past, strontium ranelate was used in the systemic treatment of osteoporosis (for example, Protelos, France), but its bioavailability is significantly reduced (by 60–70 %), depending on the patient's diet. Systemic use of strontium ranelate also has a number of serious adverse events, including indigestion, mental changes, venous thrombosis, and myocardial infarction (*Jonville-Bera et al., 2011*). *Hao et al.* study showed that it could improve osteogenic differentiation and bone formation of mesenchymal stem cells *in vivo*, as well as reduce the number and activity of osteoclasts (*Hao et al., 2015*). Because strontium acts as a dual agent, the optimal amount of strontium remains a research issue. As described in the literature, strontium levels in bioceramics range from 1 % to 100 % (*Ehret et al., 2017*). *Saint-Jean et al.* study demonstrated that low concentrations of strontium in biomaterials reduce bone resorption and stimulate bone formation (*Saint-Jean et al., 2005*). Similarly, in *Grynepas et al.* study the vertebral bone volume of rats increased by 17% (*Grynepas et al., 1996*). Also in our study, biomaterials with a small amount of strontium improved the biomechanical properties of bones compared to other groups of osteoporotic animals. In contrast, no statistically significant differences in bone mineral density were found after implantation of biomaterial with or without the presence of strontium.

Literature data have shown that higher doses of Sr have the opposite effect by inhibiting the bone mineralization process (*Grynepas et al., 1996*). Similar adverse effects were observed *in vitro* in osteoblasts, where higher doses of Sr (> 20 µg / ml) interfered with the formation of hydroxyapatite by reducing bone mineralization (*Verbercmoes et al., 2003*). Therefore, further research with

different amounts of strontium in biomaterial is needed to better understand Sr role in osteoporotic bone regeneration mechanisms.

In the present study, 5 % strontium-containing biomaterials improve bone biomechanical properties compared to other groups of osteoporotic women. In contrast, no statistically significant differences were found in bone mineral density after implantation of biomaterial with or without strontium. Better results may be obtained if the strontium concentration in the biomaterial is higher. Further research is needed with different amounts of strontium in the biomaterial to better understand their role in the mechanisms of osteoporotic bone regeneration.

When studying the effect of Riga Technical University Rudolfs Cimdins Biomaterials Innovation and Development Centre biphasic calcium phosphate bioceramics on bone tissue near the implant site (right thigh), it was necessary to find out whether this biomaterial also has any effect on bone tissue in remote areas, such as left thigh. In the performed study, the biomechanical parameters and mineral density of both thighs after biomaterial implantation were compared. No statistically significant differences were found between the ultimate strain, ultimate stress, flexure modulus and mineral density of operated and non-operated thigh samples ($p > 0.05$). From this, it can be concluded that the effect of the biomaterials on osteoporotic bone biomechanical parameters and mineral density near the implant site (right thigh) and in the distal region (left thigh) is the same, indicating a systemic effect of the biomaterial on osteoporotic rabbit bone.

Conclusion

1. The ultimate strain of osteoporotic rabbit samples after biomaterial implantation is statistically significantly lower compared to the placebo group, which indicates an improved ability of bones to resist strain forces.

2. The ultimate stress of non-operated thigh and lower jaw specimens after biomaterial implantation is statistically significantly higher compared to the placebo surgery group, which indicates an increase in bone strength. In contrast, no statistically significant differences were found in the groups of operated thigh samples.

3. The flexure modulus of operated, non-operated thigh and lower jaw specimens after biomaterial implantation is statistically significantly higher compared to the placebo group, which indicates an increase in bone stiffness properties.

4. The mineral density of osteoporotic rabbit samples after biomaterial implantation is statistically significantly higher compared to the placebo group, which indicates the systematic effect of biomaterials on the bones in experimental animals.

5. Comparing the biomechanical parameters of the operated and non-operated thigh samples and the mineral density groups after biomaterial implantation, no statistically significant differences were found, indicating the same effect of biomaterials on osteoporotic bones located far from the implantation site.

6. Strontium-containing biomaterials improve the biomechanical properties of bones compared to other groups of osteoporotic animals. In contrast, no statistically significant differences were found in bone mineral density after implantation of biomaterial with or without strontium.

7. In cross-section, the area of trabecular bone in bone samples from healthy rabbits was statistically significantly larger than in osteoporotic rabbits, which is evidence of osteoporosis in experimental groups of animals. In turn, a similar trabecular bone area for all groups of osteoporotic animals indicates a constant bone volume after biomaterial implantation.

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