

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**TOPICAL TREATMENT OF OSTEOPOROSIS BY
IONTOPHORESIS USING CALCIUM- AND
PHOSPHATE-DONATING MICROPARTICLES**

by

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1. INTRODUCTION AND BACKGROUND

1.1. Description of bone tissue

1.1.1. Components of bone

Bone is a type of connective tissue consisting of organic (collagen and non-collagenous) and inorganic components combined together to provide mechanical and supportive role in the body [1-5]. The bone is composed of inorganic mineral (70 % of weight), organic matrix and cells (25 %) and water (5 %) [2, 3].

Newly synthesized bone matrix called osteoid is essentially completely organic. Approximately 94 % of the weight of unmineralized matrix consists of collagen. Depending on the orientation of collagen fibres, bone exists in two forms types: lamellar or cortical bone and non-lamellar (trabecular or cancellous) bone [2]. Another 4 % of the weight contains non-collagenous proteins, such as glycoproteins and phosphoproteins including osteonectin, sialoproteins, osteocalcin and bone morphogenic protein (BMP), as well as different enzymes, hormones, growth factors, and other metabolites [2, 6]. Bone cells constitute only 2 % of the organic part [2].

Understanding the inorganic phases of bone is of high importance for the treatment of bone diseases, and for the development of biocompatible bone replacements as well. Three calculation models were developed by our research group in order to determine the chemical formula of bone apatites by thermoanalysis [7]. The results support the theory of the presence of distinct calcite phase in bones, along with carbonate containing calcium-hydroxyapatite. Our research also confirms that CO_3^{2-} is an important factor regarding solubility and thus biocompatibility as it dissociates during and after the combustion of collagen. This explains the observation of better properties, the faster bone forming and collagen connection of cements and prosthesis sintered in low temperature [8, 9].

Hydroxyapatite ($\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$) is generically referred to as the bone mineral component. This plate-like crystal is 20 to 80 nm long and 2 to 5 nm thick. The form of apatite presenting in human bone is quite different from that occurring in nature as it contains sodium, fluoride, strontium, lead and radium as well. In addition, it is smaller in size (10 to 40 nm), more soluble and more reactive due to its less-perfect atomic

arrangement [3, 5, 10]. The nucleation sites of bone mineral consists of more energetically favorable amorphous calcium phosphate and octacalcium phosphate crystals [10]. These precursors are formed first and transformed later to the more stable and crystalline hydroxyapatite [10, 11]. The surface area of amorphous calcium phosphate with its hydration shell facilitates the mineral exchange. The bone mineral continues to mature throughout lifetime exposing less surface area [11]. Because of this greater levels of systemic factors are needed to liberate calcium from the more stable crystals. In addition, the magnitude of stress-generated electrical charge in the form of zeta potentials drops proportionally as less mineral is exposed at the microboundary layer. The process of this maturity may contribute to the pathogenesis of osteopenia [10, 11].

1.1.2. Mineralization of bone tissue

Mineralization of bone tissue is a very complex and dynamic process. It begins 10 to 15 days after the deposition of osteoid [2, 5]. The amount of bone mineral increases immediately to 70 % of its final content but the deposition of the remaining 30 % would then take months [2]. Calcium phosphate nucleation initiates with the formation of the matrix vesicles. These vesicles are extracellular lipid bilayer-bound organelles that sprout from apoptotic hypertrophic chondrocytes and osteoblasts and from various plasma membrane cell processes [12]. Matrix vesicles contains phospholipids which have strong calcium affinity and special membrane-bound calcium pumps. During the process of mineralization, calcium ions accumulate in the vesicles and at a point of supersaturation, nucleation of mineral begins [2, 12]. During osteoid production, alkaline phosphatase concentration increases. Its activity increases the local concentration of phosphate, thus it facilitates the deposition of hydroxyapatite on the inner surface of the vesicle [13]. This leads to the destruction and dissolution of vesicle membranes so hydroxyapatite crystals will be exposed to the extravascular compartment [13]. The crystals then move toward collagen fibrils by chemotaxis and bind to them. Mineralization extends over the collagen matrix parallel with the fibers. The arrangement of the collagen matrix determines the orientation of the bone mineral crystals [1, 10, 13].

The calcification in the extravascular milieu is inhibited by glycosaminoglycans that modulate the advancing mineral front; by tighter packing of collagen fibers resulting in limited access of phosphate ions to interfibrillar nucleation sites; and by crystallization inhibitors, such as pyrophosphates. When mineralization is halted, a thin layer of osteoid is

left between the lining cells and the mineralization front, and the syncytium is established. This cellular canopy must be retracted to reinitiate the remodeling process [10, 12].

The complex nature of bone and the sophisticated process of mineralization make bone an optimal tissue that serves as a structural organ and as a mineral reservoir as well.

1.1.3. Mechanical properties of bone

The success of bone as a structure depends on mechanic properties, such as stiffness, strength, toughness and the ability of structural adaptation. The mechanic strength of mature osteonal bone is greater than of immature bone which is composed of osteons that are only partially mineralized [2, 14, 15]. The normal value of the stiffness of the haversian bone is about 17 gigapascals in the longitudinal direction, 22,5 gigapascals in the transverse direction and 3,3 gigapascals in shear [14]. The degree of mineralization or porosity compromises the stiffness of the bone and lowers the elastic modulus. However the decreased stiffness can be compensated by changing the morphology (e.g., periosteal expansion) resulting an 'effective' modulus. The major component of the strength of bone is derived from its composite that works synergistically to avoid yield. Strain is a unit of change in length divided by original length. The yield strain or the degree of deformation of bone reached at the point where the bone does not elastically recover (the tissue damaged irreversibly) is 7000 microstrain or 0,7 percent change in length. The ultimate strain at which the bone actually fractures is 15000 microstrain. During flexion individual lamellae arranges relatively to adjacent lamellae in a way that enable dissipating energy and minimizing strain levels within the material, thus allowing the entire system to react in a more elastic manner [2, 14, 16-18].

Bone needs to be not just stiff to resist deformation but also tough to prevent crack propagation. To adapt to changes in the physical demands placed on it, the skeleton responds by changing its mineral content and by altering its shape and morphology achieved by functionally regulated alterations in bone mass and architecture [16, 17].

The course and balance of bone remodeling can be affected by mechanic functions. Contrary it appears that minimizing strain is not the ultimate goal of adaptation. Instead the combination of skeletal morphology and the locomotion character strives to generate a certain type of strain in a beneficial level [16, 17, 19, 20].

Bone remodeling is continually influenced by the level and distribution of the functional strains within the bone [14, 20-22]. The alterations in bone mass, turnover, and internal

replacement are sensitive to changes in the magnitude, distribution, and rate of strain generated within the bone tissue [14, 17, 20]. Bone mass is influenced by strain situations by short periods of particularly osteogenic activity rather than by the strain situation experienced during predominant activity or by the fatigue damage [14, 17, 20].

A change in the bone's level of activity in turn alters the magnitude of stress-generated electric potential charge at the bone-fluid interface. The deformation of the skeleton caused by activity generates a flow of the fluid in the haversian system. The ionic constituents of the fluid interact with the charged nature of the mineral and generate electrokinetic potentials [23]. The intracortical syncytium might be the key regulator of bone cell activity. The stress-generated electric potential may act to effect proliferation of osteoprogenitor cells, catalyze production and mineralization of the ECM, and can affect both differentiation of osteoblasts and osteoclasts [24, 25]. Even the signal transduction pathway of transforming physical information to something the cell population can perceive and respond to is just partly known, the osteogenic potential of physical stimuli cannot be ignored.

1.1.4. Physiology and pathophysiology of bone

Bone is a complex tissue that regulates its mass and architecture in order to meet two important responsibilities: its structural and metabolic function. The skeleton protects vital organs and facilitates locomotion. On the other hand, it serves as a mineral reservoir containing 99 % of total calcium and 85 % of total phosphorus of the body. This dual responsibility creates competing stimuli on the anabolic and catabolic regulation of the bone [2].

Abnormalities of ion balance or the endocrine system, or renal failure may lead to impaired structural integrity of the skeleton, while regulatory networks try to maintain calcium and phosphorous homeostasis. Due to altered functional demand, the structural adaptation of the skeleton can proceed apace, even ignoring consequences of rapid fluctuations in serum calcium levels [2, 26, 27].

Despite the different regulatory mechanisms serving metabolic and structural needs, skeletal integrity and viability are decisively dependent on maintaining an intricate balance between them. This balance is a result of the complex cellular mechanism of formation and resorption of the bone. The constant modeling and remodeling process is achieved through the network of bone cells: osteoblasts, osteocytes and osteoclasts. These cells mediate the remodeling balance and they provide the machinery for the maintenance of calcium

homeostasis [1-4, 28, 29].

By the age of 30, humans attain a mature skeleton and bone mineral density (BMD) peaks. Maximum BMD is controlled by hereditary factors [1, 26, 27, 30, 31]. After this peak, bone mineral content (BMC) continuously declines, while skeletal susceptibility to fracture increases. Normal sex steroids are required to attain peak bone mass, the deficiency of these hormones increases the rate of mineral loss [32]. The importance of vitamin D, parathyroid hormone (PTH), calcitonin, transforming growth factor β (TGF- β) and insulin-like growth factor 1 (IGF-I) in regulating bone turnover has been well established [1, 30, 33-35]. Certain medical conditions, such as corticosteroid treatment or many chronic comorbidities, as well as aging also affect bone density [1, 4, 36].

Any change in the balance between bone resorption and formation ultimately results in significant loss or gain of bone tissue. High bone turnover with increased bone resorption or decreased bone formation or both results in reduced bone mineralization and abnormal bone microarchitecture. The thinning of the bone structure and compromising bone strength leads to a greater propensity to fracture [4, 17, 36].

1.2 Osteoporosis

1.2.1. Definition

Osteoporosis is characterized by low BMD and a deterioration of bone microarchitecture that reduces bone strength and thus leads to increased fragility and susceptibility to fractures. The main characteristic of osteoporosis is the reduction of bone mineral and bone matrix at the same extent thus maintaining normal mineral-to-matrix ratio. The structural bone deficit may be attributed to enhanced resorption and/or decreased bone formation [1, 4, 5].

1.2.2. Epidemiology

Osteoporosis is one of the most common chronic disorders and the most common metabolic bone disease. Today, more than 40 million people in the United States and more than 750 million people worldwide (about 10.6% of the population) either are at high risk due to low bone mass or already have osteoporosis. It can strike at any age, although the risk for the disease increases with years of age [37-49].

Over 50 years of age, 40 % of women and 13 % of men develop one or more osteoporotic fractures in their lifetime. These occur most commonly in the spine, in the hip and in the wrist. Fifty percent of people who fracture a hip will be unable to walk without assistance. Within the first year after fracture, 12-24 % mortality rate in women and 30 % mortality rate in men are observed. Half of these patients require long-term nursing home care [4, 38, 39]. In Hungary, the number of osteoporosis-related hip fractures is approximately 12,200 patients per year in the population aged 50 to 100 [4, 38, 42, 48].

These figures should continue to grow exponentially as the elderly population of industrialized nations increases over the years. Thus, osteoporosis has become a global health issue referred to as ‘silent epidemic’. It is a major medical and economical burden for both the individual and the society [38, 39, 42, 50].

1.2.3. Pathophysiology of osteoporosis

Bone is undergoing constant remodeling. Osteoporosis is a result of an imbalance between bone resorption and formation.

Low peak bone mass and rapid bone loss after menopause, are mechanisms that have been associated with primary postmenopausal osteoporosis. An acceleration of bone loss usually occurs over 5 to 8 years with an annual loss of 2-3% of trabecular and 1-2% of cortical bone [1, 2, 29]. Metabolic changes e.g. estrogen loss and affection of local factors can increase osteoclast number and activity that leads to greater bone resorption than formation resulting in loss of bone tissue [1, 4, 5, 51-53].

Estrogen loss in postmenopausal osteoporotic women has the potential to influence ischemia-reperfusion induced inflammatory activation [54, 55]. Szabó et al. set out an osteoporotic rat model where the consequences of a long-term female hormone deficiency could adequately be estimated, furthermore the effects of chronic estrogen supplementation on the post-ischemic local and systemic inflammatory reactions were assessed [56, 57]. Based on the results it can be suggested that ovariectomy does not predispose the periosteal microcirculation to enhanced local leukocyte activation and inflammatory cytokine release. Thus, osteoporosis itself does not increase the magnitude of the limb ischemia-reperfusion-associated periosteal inflammatory reaction. Chronic estrogen supplementation, however, significantly ameliorates the microcirculatory consequences of tourniquet ischemia [58].

Estrogen deficiency in postmenopausal women is associated with the release of pro-inflammatory and bone-resorbing cytokines, such as tumor necrosis factor α (TNF- α),

interleukin 1 (IL-1) and IL-6. To date, the most important network that regulates bone resorption is the receptor activator of nuclear factor kappa B (RANK) – RANK ligand (RANKL) – osteoprotegerin (OPG) pathway [1, 28, 59-62]. These molecular pathways will not be discussed in more detail.

In addition, a number of genetic, nutritional and lifestyle-related risk factors predispose to the development of osteoporosis. These factors include, among others, Caucasian race, type I collagen A1 (*COL1A1*) gene polymorphism, excessive alcohol intake, physical inactivity or impaired vision.

By age 65, men and women tend to be losing bone tissue at the same rate and this more gradual bone loss continues throughout life. Factors contributing to age-related bone loss also include impaired calcium intake, compensatory rise of PTH levels, increased bone resorption over formation. With increasing age, there is a significant reduction in bone formation mostly due to a shift from osteoblastogenesis to predominant adipogenesis in the bone marrow. The etiologic factors that lead to senile osteoporosis still are not totally clear [63].

Secondary osteoporosis has been associated with endocrine abnormalities including thyroid diseases, glucocorticoid excess, hyperprolactinaemia; diseases affecting bone marrow, such as multiple myeloma, leukemia, Gaucher's disease; gastrointestinal diseases; rheumatic disorders, such as rheumatoid arthritis, ankylosing spondylitis. Various medications, such as heparin, methotrexate, lithium may also induce bone loss [1, 4, 31, 64-66].

1.2.4. Diagnostic procedures

Osteoporosis may be diagnosed by history taking, clinical signs and assessment of BMD and bone turnover by radiographic and laboratory methods [5, 52].

1.2.4.1. Assessment of bone density

In the daily clinical practice, dual-energy X-ray absorptiometry (DEXA) is used to assess BMD. Bone density evaluation incorporates the attenuation of soft tissue and bone by X-rays in order to calculate BMD. This method is safe, precise and is able to detect small changes over time. Moreover, the patient is exposed to very low radiation. In order to detect

the reduction in BMD, specific parameters are calculated. The most frequently used parameter is T-score that express the difference of BMD in the patient compared to that in young healthy controls. There is an inverse relationship between BMD and the extent of fracture risk [52, 67-70].

Using ultrasonography (US), the amplitude dependent speed-of-sound (AD-SoS) technique provides information on the mechanical properties of bone including elasticity-qualities that are strongly associated with bone strength. Although it is a radiation-free technique, its reproducibility is not as good as that of DEXA [71-73]. By the use of quantitative computed tomography (qCT), the loss of trabecular or cortical bone can be measured directly and separately, yet, qCT entails high radiation exposure and time, and is more expensive than DEXA [70, 74, 75]. US and qCT are used in osteoporosis research rather than in the everyday clinical practice.

The World Health Organization (WHO) has established a set of criteria for osteoporosis based on bone density. Osteopenia is defined by T-score between -1 and -2.5; osteoporosis is defined by T-score less than -2.5; and established osteoporosis is defined by a T-score less than -2.5 and the presence of an osteoporotic fracture [70, 76].

1.2.4.2. Radiography

In advanced osteoporosis, signs of demineralization and evidence of bone fractures can be detected by simple X-ray imaging. Radiographs may identify signs of secondary causes of bone loss, such as subperiosteal resorption in hyperparathyroidism, metastatic osteolytic changes, bone infarcts or pseudofractures in osteomalacia. Thus, DEXA and modern laboratory techniques have not fully replaced the use of conventional radiography [52, 70, 76].

1.2.4.3. Laboratory markers of bone turnover

Sensitive biochemical markers are used to analyze changes in bone formation and resorption at any time-point. Osteocalcin produced by osteoblasts, as well as bone-specific alkaline phosphatase (BSAP), procollagen I carboxyl- (PICP) and amino-terminal peptides (PINP) are markers of bone formation. Markers of bone resorption including urinary and serum pyridinoline (PYD) and deoxypyridinoline (DPYD) collagen cross-links, cross-linked N (NTX) and C telopeptides for type I collagen (CTX) are derived from the degradation of mature collagen. The release of these markers increases with menopause and remain high in disorders characterized by accelerated bone turnover [52, 53, 70, 77-79].

1.2.4.4. Evaluation of secondary osteoporosis

Various secondary causes of bone loss should also be excluded. In the daily practice, determination of calcium, phosphate, supersensitive thyroid stimulating hormone (sTSH), 25-hydroxyvitamin D and PTH serum levels, urinary calcium and creatinine levels, full blood cell count, liver and kidney function tests, erythrocyte sedimentation rate (ESR) and serum protein electrophoresis are needed for differential diagnosis [4, 52, 59, 65, 80].

1.2.5. Treatment of osteoporosis

1.2.5.1. Drug therapy

The major goal of osteoporosis treatment is the prevention of fractures by reducing bone loss, preferably by increasing bone density and strength. Early detection and timely initiated treatment can decrease the risk of fractures [5, 43, 51, 52, 81-84].

To date, no treatment modalities that stop bone loss and cure osteoporosis have been developed. Therefore, life-long prevention is very important. Lifestyle changes, including quitting cigarette smoking and excessive alcohol intake, physical activity, balanced diet with adequate calcium and vitamin D intake can reduce bone loss and prevent future fractures [51, 52, 85-87].

Several drugs not to be discussed in detail have been introduced to the treatment of osteoporosis. Antiresorptive agents include oral and parenteral bisphosphonates including alendronate, risedronate, ibandronate and zoledronate, as well as the selective estrogen receptor modulator (SERM) raloxifene, HRT and the anti-RANKL monoclonal antibody denosumab. Bisphosphonates inhibit the resorption of bone by enhancing osteoclast apoptosis. Raloxifene is an oral SERM that has estrogen-like actions on bone and anti-estrogen effects on the breast and uterus. HRT has long been used to treat osteoporosis, however, long-term HRT may increase the risk of cancer and cardiovascular disease. Therefore the use of SERMs and other anti-resorptives rather than HRT is recommended. Denosumab is a fully human monoclonal antibody, used for primary osteoporosis as well as for treatment-induced bone loss and bone malignancies. Bone formation can be stimulated by teriparatide (PTH 1-34). This bone anabolic agent stimulates bone formation and activates bone remodeling by many ways. Strontium ranelate is an unusual drug that it increases bone formation and reduces bone resorption at the same time thus re-balancing bone turnover [1, 4, 51, 52, 81, 88-122].

Despite the success and effectiveness of these therapies, an osteoporotic fracture occurs in every 30 minutes in the European Union [39-41, 48]. Thus, the question arises whether osteoporotic women are more susceptible to postoperative or post-traumatic inflammatory bone complications than postmenopausal women receiving estrogen replacement therapy [54, 55]. To answer this question an osteoporotic rat model was set out where the consequences of a long-term female hormone deficiency can adequately be estimated [56, 57]. In this study, we have demonstrated that ovariectomy does not predispose the periosteal microcirculation to enhanced ischemia-reperfusion-induced inflammatory complications such as local leukocyte activation and inflammatory cytokine release. And further, the mitigating effect of chronic estrogen therapy on the systemic and PMN-driven local periosteal inflammatory reactions is independent of its antiporotic effects [58].

In addition to the high incidence of osteoporotic fractures, anti-osteoporotic drugs may also have important side-effects that limit their use in elderly patients and in those with comorbidities [51, 81].

Therefore there is a need for alternative therapies, such as non-medicinal treatments including physiotherapy that can be included in the complex management of osteoporosis [123].

1.2.5.2. Physical therapy in osteoporosis

Physical activity and exercise are important for the preservation of musculoskeletal health. Exercise-mediated alterations in the Wnt/ β -catenin signaling pathway may facilitate the maintenance of bone mass and density. The best type of exercise for postmenopausal women to improve BMD is progressive resistance training, either alone or in combination with other interventions. In the elderly, physical activity and exercise only have minimal effects on BMD, rather fall prevention and strength training should be the focus in this age [124].

Balneotherapy may also have positive effects on the bone, in part, by the transdermal diffusion of minerals into the body [125, 126]. In these studies, the agents were delivered into the blood circulation.

Electrotherapy, such as iontophoresis (IOP) could be applied in cases of localized osteoporosis, such as reflex sympathetic dystrophy (RSD, also known as Sudeck's atrophy, algoneurodystrophy or complex regional pain syndrome) or to heal bone fractures. Some experimental reports on local transdermal administration of magnesium, vitamin D,

dexamethasone, PTH 1-34, calcitonin or even Chinese herbal medicines to the bone using IOP have been published [127-133].

These preliminary investigations triggered our studies that included the development of a new IOP device and the method of local transdermal administration of calcium and phosphate ions into the bone by IOP.

1.3. Iontophoresis

1.3.1. Definition of IOP

The term iontophoresis is simply defined as ion transfer (ionto = ion; phoresis = transfer). IOP is a technique using a small electric charge to deliver a chemical drug through the skin. This process is a non-invasive method of propelling high concentrations of a charged substance transdermally by repulsive electromotive force using electrodes with small electrical charge. IOP is a well classified method for transdermal drug delivery relying on active transportation within electric field. The dominant forces of this transport are electromigration and electroosmosis, which measured in units of chemical flux (commonly in $\mu\text{mol}/\text{cm}^2\text{h}$) [123, 132, 134, 135].

1.3.2. Basic principles of IOP

In order to deliver the ions into the tissues, a direct galvanic current (DC) needs to be employed. The substance to be driven into the tissues needs to be ionic in nature, and should be placed under the electrode with the same charge. The positively charged electrode called the anode, will repel a positively charged chemical, whereas the negatively charged electrode, called the cathode, will repel a negatively charged chemical into the skin. Ions are delivered via the pores of the skin rather than through the stratum corneum which has a high resistance, therefore limited current can pass through it. The pores have lower resistance, so a greater passage of current is possible. The penetration of the ions is the greatest in the region of the pores. The penetration of the substance through the skin is in proportion to the current magnitude, but the substance makes a deposit below the stratum corneum, acting as a depot. Onward migration to deeper tissues can be achieved mainly by diffusion. It is also suggested that if there is a strong vasodilatation in the capillaries of the skin, a less effective

diffusion will be possible to deeper tissues because the increased local flow serves to dilute the subepidermal deposit [123, 134, 135].

During the process negatively charged ions (chloride ions) of the skin transit to the anode, therefore under the positive electrode acid accumulation is probable. Alkaline would accumulate under the negative electrode, because of the transit of positively charged sodium ions of the skin [134-136].

There have been numerous instruments designed for iontophoresis. Several of them are for patient home use (e.g. for the treatment of hyperhydrosis). The electrode patches of these modern multifunction devices are preconfigured and the instrument delivers a smaller current than normally used in the clinics (typically 0.1mA). For the proper treatment the skin of the treated area should be cut free and cleaned. All electrode pads should be soaked in solution prior to application, dry electrodes are inappropriate. Electrodes can be special pre-gelled electrodes or standard metal electrodes. If metal is used the substance should be applied to the wet or damp gauze between the metal electrode and the skin surface. Commonly, the negative electrode is likely made larger relative to the positive electrode to avoid skin irritation. Current intensity usually used for IOP is up to 5 mA, the available evidences suggest that low current intensity appears to be the most effective for delivery [132, 134-136].

Constant current is preferable to constant voltage, thus, whatever changes occur in terms of skin resistance, the magnitude of the applied current will not exceed the preset level. The current density (measured in mA/cm²) is an important factor in IOP. If the current density reaches a too high level, tissues can be damaged. It has been suggested that a current density of no more than 0.5 mA/cm² should be applied at the cathode and 1.0mA/cm² at the anode. If a current of 2 mA is delivered using an electrode of 6 cm², the current density will be 0.33 mA/cm², which is safe at either the positive or negative electrode [135, 136].

In summary, the optimal parameters for IOP are: direct current type with the intensity of 1 to 4 mA, 20 to 40 minutes for duration of treatment and 40 to 80 mA/min as total current delivered. Low ionic concentration (up to 5 percent) of the applied substance is preferred to be used, though higher concentration of the applied agent does not increase the amount of the drug being delivered (typically 2 to 5 percent) and does not serve to increase the effectiveness of the therapy. The effects of the treatment are attributed mainly to the delivered ions and not to the current, but this theory has not yet been fully established [134-136].

1.3.3. Iontophoresis in osteology

Bernard, a French dentist, constructed a device on the principles of electrophoresis, which was suitable to treat human patients. Bernard's device provided several forms of applicable current including monophasic (MP), biphasic (BP), short period (SP), long period (LP) and direct current (DC), as well as their combinations [135].

The first IOP device employed for human therapy was developed and patented by the Hungarian scientist Dezső Deutsch in the 1930's [137]. In the 1950's, Riesz, a Hungarian researcher, developed the technique of "calcium electrostasis" and used this method to treat delayed callus formation and reflex sympathetic dystrophy (Complex Regional Pain Syndrome, CRPS) [138-141]. Topical IOP and calcium ionostasis have been used in bone fractures, delayed union and algodystrophy for decades [142-144].

IOP has also been used to deliver various chemicals and drugs into humans for the treatment of osteoporosis [127, 129, 133]. Galvanic current IOP using calcium, phosphorus and vitamin D enhanced the speed of bone healing in dogs [129]. Magnesium iontophoresis enhanced mineralization of teeth and bones [128, 145]. Pulsatile transdermal IOP administration of human parathyroid hormone (1-34) prevented bone loss in ovariectomized animals [130]. Transdermal salmon calcitonin IOP has also been developed and investigated on rats [131].

1.3.4. Development of natural-based microparticles and a novel three-electrode IOP apparatus suitable for simultaneous calcium and phosphate iontophoresis

1.3.4.1. Experiments of calcium iontophoresis

In the 1990's a new method for the local administration of calcium ions by IOP has been invented and developed by our research group, Pap et al. [146, 147]. Transdermal delivery of Ca^{2+} through different membrane models was investigated by spontaneous and IOP diffusion, using Ca^{2+} -containing compounds with different dissociation constants. These experiments were carried out on bentonite (a natural mineral clay with a large ion exchange capacity), previously enriched in calcium ions in its lattice.

Calcium ion transport through the pig skin has been investigated in vitro in diffusion cells applying IOP. The best Ca^{2+} transmission efficiency was achieved using short period (SP) current: the amount of the calcium ions penetrating through the pig skin by IOP was 5

to 10 times higher than that of the passive transport. The results of these *in vitro* studies opened a new field of the application pattern of IOP [146, 147].

Afterwards, calcium IOP was performed on porcine tissues *in vitro*. The porcine tissue system containing skin, fat, muscle and bone was found the most appropriate for these experiments. The delivery of calcium ions into these tissues was assisted by the electrophoretic method described above. As a result of bentonite IOP, calcium concentrations of the treated bones significantly increased in an average nearly 1.8-fold [148].

1.3.4.2 Development of natural-based microparticles suitable for simultaneous calcium and phosphate iontophoresis

After the favorable results of calcium iontophoresis, specially modified forms of calcium- and phosphate-donating microparticles appropriate for transdermal IOP delivery, were developed by our research group, which method is protected by patent [149].

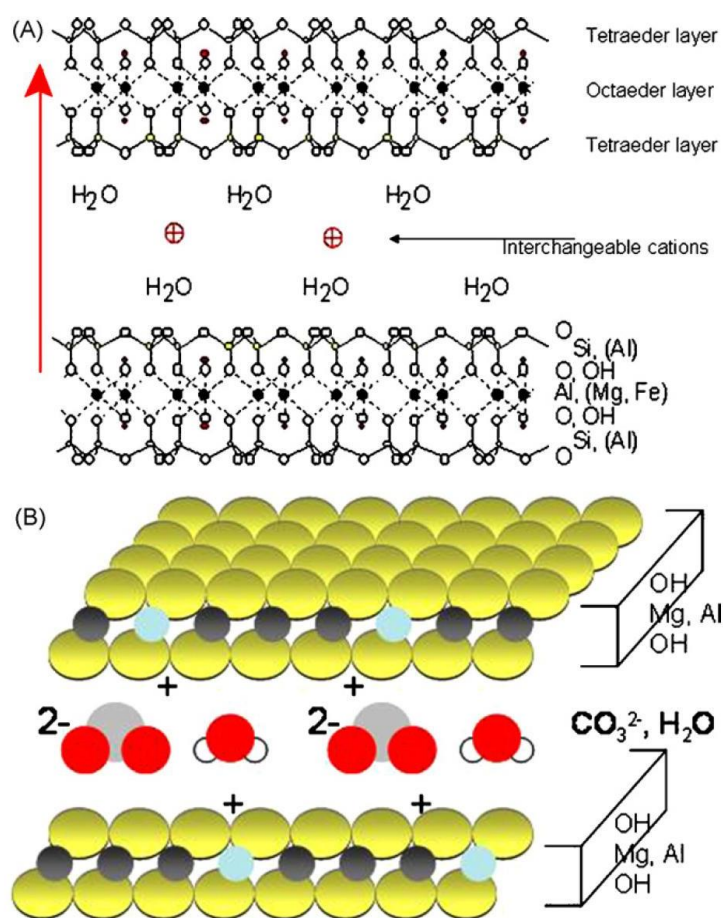


Figure 1. Elemental cell of montmorillonite (A) and hydrotalcite (B)

Montmorillonites including bentonites with silicate layer lattices are of highly different qualities depending on many geological factors [150, 151]. These compounds, because of their toxic components, need to be purified before *in vivo* use [152, 153].

The investigators developed a novel molecule composed of silicate layer lattices as Ca^{2+} source, which has a stable composition and is free of toxic trace elements [146, 147] (Figure 1A). This molecule was found suitable for the planned IOP experiments. The optimization of this compound is currently underway and protected by patent [149].

Hydrotalcite is a natural lattice layer, containing phosphate ions. This microparticle can also be produced synthetically, using high-purity components as detailed in a Japanese patent [154-156]. Our new protocol developed in order to modify the phosphate donating microparticle to be more applicable for *in vivo* use slightly differs from the original one above [149] (Figure 1B).

1.3.4.3. Development of a novel three-electrode IOP apparatus

A novel three-electrode IOP apparatus adequate for simultaneous calcium and phosphate iontophoresis had also been developed [149] (Figure 2).

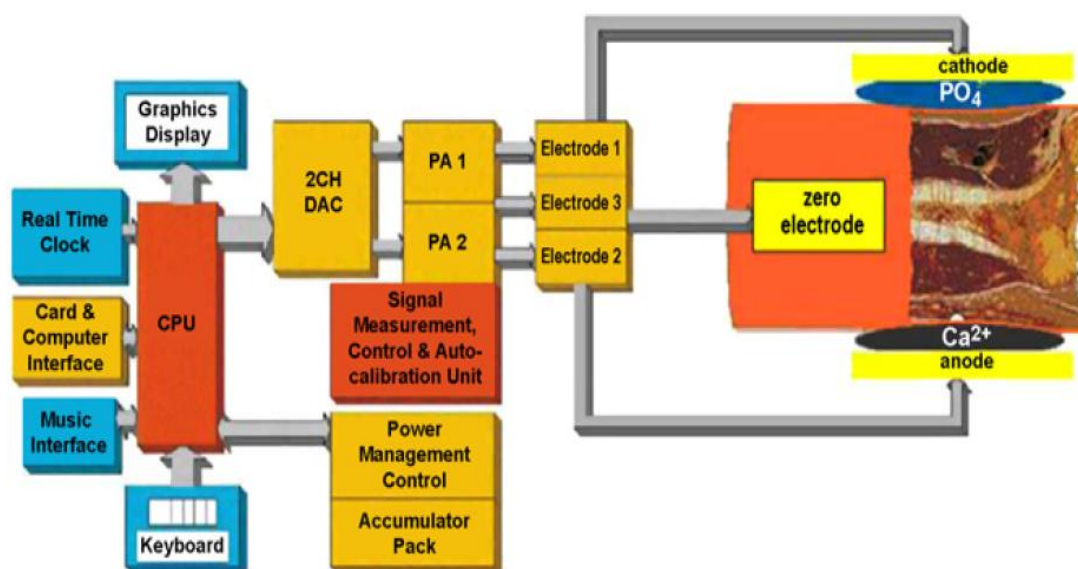


Figure 2. Simplified block diagram of the iontophoretic instrument and the scheme of treatment protocol

The major advantage of this instrument supplied by a one-phase network is that the reference electrode and the anode, as well as the reference electrode and the cathode are

forming two separate circuits. The voltage of the anode and cathode can be adjusted separately, thus the migration of positive and negative ions from the Ca^{2+} and PO_4^{3-} sources can be independently controlled on the electrodes. The positive and negative electrodes must be both placed at an angle of 120° from the marked site. The reference electrode, which is on zero potential, brings down the current exiting the instrument to zero and also allows an optimal focusing of voltage.

With the innovations detailed above, a more effective ion delivery can be performed. Besides, no earth current could appear at any part of the body.

After the development of the calcium- and phosphate donating molecules and the new IOP apparatus [157, 158], we used this instrument and these techniques for further *in vitro* and *in vivo* animal studies.

2. RESEARCH AIMS

The aim of my Ph.D. study was to perform first in the world *in vitro* investigations, *in vivo* and *postmortem* experiments for the setting of the novel calcium and phosphate iontophoresis treatment, for the detection and clarification of its effects, safety and efficacy on animal bones.

The main issue to be answered was whether the novel iontophoretic apparatus and ion donor microparticles specifically developed for this purpose are applicable for calcium and phosphate transdermal delivery into bone.

Our specific aims were as follows:

- To investigate *in vitro* whether calcium and phosphate ions are delivered into the target porcine bone by the novel iontophoresis and to determine the extent of calcium and phosphate concentration changes in bone
- To investigate *in vivo* the effects of the novel iontophoresis in terms of changes in bone density, biomechanical parameters and calcium and phosphate ion concentration of rat bone
- To compare the *in vivo* effects of the novel iontophoresis with estrogen supplementation on osteoporotic rat bones

3. MATERIALS AND METHODS

3.1. Materials for simultaneous calcium and phosphate iontophoresis

3.1.1. Calcium and phosphate donor molecules

We used specially modified calcium-bentonite and hydrotalcite layer lattices as calcium and phosphate source developed [149] as described above (Figure 1). In brief, the calcium donor molecule was composed of toxic-free silicate layer lattices with a stable composition and had found suitable for iontophoresis.

The phosphate donor molecule used for the experiments was a synthetically produced and modified hydrotalcite based lattice layer containing phosphate ions. This form had found before the most applicable for iontophoretic use.

3.1.2. Iontophoretic apparatus

A novel three-electrode IOP apparatus described previously was used for all of the experiments [149] (Figure 2). This instrument is adequate for simultaneous calcium and phosphate iontophoresis as it forms two separate circuits with the help of the third zero electrode. Thus the voltage or current of the electrodes (anode and cathode) is separately adjustable. As a consequence, the migration of positive and negative ions from the Ca^{2+} and PO_4^{3-} sources into the treated area can be independently controlled. The reference electrode, which is on zero potential, allows an optimal focusing of voltage.

The instrument was constructed to comply with strict safety requirements.

3.2. Elemental analysis

3.2.1. Reagents and instruments

All reagents used in these studies were of ‘analytical reagent’ grade unless otherwise stated. All the experiments were carried out at room temperature.

Triple deionized water was used for preparing sample solutions, which was produced by using a Milli-Q RG apparatus (Millipore, UK). 65% nitric acid and 30% hydrogen-peroxide

were used for the wet digestion of the samples. For the microwave digestions a MILESTONE MLS-1200 Mega MDR apparatus (Milestone Inc., Italy) was utilized.

For the calibration of the atomic absorption spectrometer 1000 mg/L Ca MERCK standard and for the calibration of spectrophotometer instrument 1000mg/L PO_4^{3-} MERCK standard was diluted.

For the determination of phosphate concentration L-(+)-ascorbic acid and a special phosphate reagent consisting of amidosulfanic acid ACS reagent (Sigma-Aldrich), ammonium molybdate tetrahydrate puriss p.a. (Sigma-Aldrich), antimon(III)chloride ACS reagent (Sigma-Aldrich) and L-(+)-tartaric acid puriss reagent (Fluka) were used.

3.2.2. Tissue preparation for analysis

Microwave assisted digestion of the samples were performed as wet digestion under atmospheric pressure was not complete. 0.5 grams of the solid samples, 3 ml concentrated nitric acid and 0.5 ml hydrogen-peroxide were blended in Teflon vessels and the tissue samples were digested for 5 minutes at a power of 300W followed by 5 minutes at power of 600W. After the microwave-assisted digestion, samples were dissolved in triple deionized water filling up to 10 cm³ per sample [159].

3.2.3. Sample analysis

Calcium concentration of the sample solutions were assessed by flame AAS (Unicam SP 1900 spectrometer).

The phosphate content was analyzed by Hach DR 2000 UV-Vis direct reading spectrophotometer. Before measurements, 0.25 ml ascorbic acid (10%) and 1 ml phosphate reagent containing ammonium molibdenate were added to 25 ml of each digested solution [159-161].

In order to avoid potential bias, biomechanical and elemental analyses were performed in a blinded fashion using coded samples.

3.3. *In vitro* experiments of simultaneous calcium and phosphate IOP on porcine tissues (Study 1)

Two *in vitro* experiments: a preliminary and an extended study were performed. The main difference between the experiments was the number of samples. Moreover, in the second case more purified calcium and phosphate donating molecules were used and a safer, newer version of the iontophoretic apparatus (but with the same construction as before) was applied.

3.3.1. *Tissue specimens*

For *in vitro* studies pig back limbs were used. Each tissue blocks prepared for these studies contained skin, adipose, muscle and bone tissues and were 5 cm x 10 cm x 5 cm in size.

Tissue samples were divided into three groups in both *in vitro* studies: Group A to C refers to the preliminary calcium and phosphate IOP experiment, and Group 1 to 3 refers to the extended *in vitro* studies of the calcium and phosphate IOP.

Each groups contained six samples in preliminary studies and fifteen samples in the extended experiments.

Tissues in Group A and Group 1 served as negative controls as these samples were untreated. Tissues in Group B and Group 2 were treated by iontophoretic current without adding any calcium- or phosphate-donors in order to test the effects of current on the porcine tissues. Finally, tissues in Group C and Group 3 were treated by the novel IOP method including the use of calcium and phosphate donating molecules.

3.3.2. *IOP treatment*

The prepared porcine tissue was placed into a plastic box specially made for these experiments. The anode and the cathode were applied to the skin at the two ends of the sample (Figure 3). During the process, 9.5 mA current intensity was produced in both circuits by the three-electrode apparatus. The time of treatment was 40 minutes. This current removes calcium and phosphate ions from the particles by their positive and negative charges. Ca^{2+} and PO_4^{3-} molecules were applied to the anode and cathode, respectively.

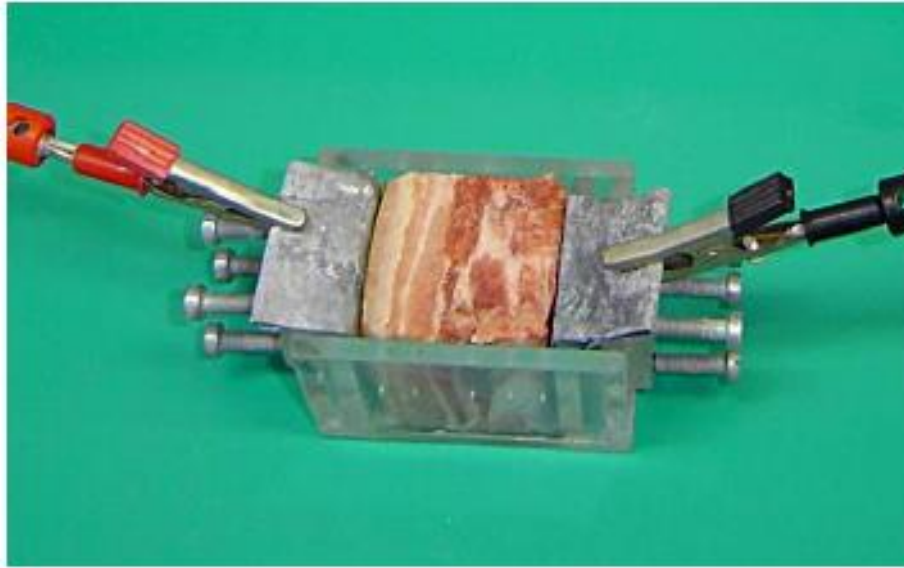


Figure 3. In vitro tissue system. Prepared porcine tissue is placed into a plastic box during iontophoresis treatment

3.3.3. Preparation of porcine tissues for analysis

After treatment, five samples were taken from the tissue block: skin samples from the anode and cathode sides, muscle samples from the anode and cathode side and bone tissue sample. The same five samples were taken from controls.

3.4. In vivo experiments on sham-operated and ovariectomized animals (Study 2 and Study 3)

Two series of *in vivo* experiments were performed. In the preliminary *in vivo* study (Study 2) only bone density changes of the tibia were detected as presenting the effects of calcium and phosphate iontophoresis on rats.

In the extended *in vivo* study (Study 3) besides long-term follow-up density changes, postmortem biomechanical experiments and elemental analyses were also performed. In this case the effect of iontophoresis was compared with of chronic estrogen therapy.

3.4.1. Experimental animals

In vivo experiments (Study 2 and Study 3) were carried out on Sprague-Dawley female rats at 12 weeks of age [162]. The average body weight of the animals was 200 grams. The

animals were housed in an environmentally controlled room with a 12-hour light-dark cycle. They were supplied with commercial rat chow (Charles River, Wilmington, MA) and tap water *ad libitum*. The animals were randomly assigned into one of the experimental groups detailed later on.

All procedures used in this experiment were approved by the Animal Welfare Committee of the University of Szeged and were performed in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals).

3.4.2. Surgical procedures

Animals were divided into two groups (ovariectomized and sham-operated) by a random manner.

Experimental animals were anesthetized with intraperitoneally administered combination of ketamine and xylazine (25 mg/kg and 75 mg/kg, respectively). Median laparotomy was performed under anesthesia with sterile conditions.

As ovariectomy the connection of the Fallopian tubes was cut between hemostats, the ovaries were removed, and then the stumps were ligated. Thereafter, the abdomen was filled up with warm sterile physiological saline solution and the abdominal wall closed in two layers [163].

Sham-operated (SH) animals underwent the same procedures except for the manipulation of the genital organs.

3.4.3. Quantitative ultrasound bone densitometry (QUS)

Under ketamine-xylazine anesthesia as described above, bone density measurements were performed at the left tibia. DBM-Sonic 1200 (IGEA, Carpi, Italy) ultrasonic bone densitometry device was used for the experiments.

The values were expressed as changes in the average of AD-SoS. After calibration the AD-SoS values of the soft tissues (muscle and skin) were determined, and the system deducted this value from the bone density [164-169]. The AD-SoS values were calculated by a computer software embedded into the densitometry instrument. The average of five measurements was used at each time points of the measurements.

In the proximal tibia statistically significant density alterations were observed 21 weeks after ovariectomy (at the age of 33 weeks of the animals), thus this location was used for further QUS measurements.

Densitometry measurements were performed at the age of 8, 9, 12 and 14 months of the rats in the preliminary (Table 1.), and at the age of 36, 39 and 45 weeks in the extended *in vivo* study (Figure 4).

3.4.4. IOP treatments

In both *in vivo* studies (Study 2 and Study 3) the novel, three-electrode IOP treatments (described above) were started at 8 months of age of the animals (day 0) and repeated 4 times (on days 2, 4, 11 and 18) (Table 1. and Figure 4).

During the treatment, the positive and negative electrodes were applied onto the skin at the two sides of the left hind limb at the proximal tibia, and the third electrode as reference was placed proximally to the other two ones. The region of interest for topical treatment (where bone loss had been detected by bone densitometry before) was marked on the skin.

Time of treatment was 30 minutes and 9 mA current was used in both circuits. During the process the experimental animals were under ketamin HCl and xylasine HCl anesthesia.

The instrument was constructed to comply with strict safety requirements.

Table 1. The scheme of preliminary *in vivo* study (Study 2) on rats

	Age: 3 months	Age: 8 months t = day 0	Age: 8 months t = day 2,4,11,18	Age: 8 months t = day 20	Age: 14 months t = day 150
Group 1 (n=6)	SH	QUS	IOP	QUS	QUS
Group 2 (n=6)	OVX	QUS	IOP	QUS	QUS
Group 3 (n=6)	SH	QUS	-	QUS	QUS
Group 4 (n=6)	OVX	QUS	-	QUS	QUS

Abbreviations: SH: sham-operated, OVX: ovariectomized animals, QUS: ultrasonographic measurements, IOP: iontophoresis

3.4.5. Experimental design

3.4.5.1. Preliminary *in vivo* study on rats (Study 2)

IOP treatment was carried out 5 times (on day 0, 2, 4, 11, 18) on sham-operated (SH) and ovariectomized (OVX) animals too (Group 1, SH+IOP and Group 2, OVX+IOP). Group 3 and Group 4 served as SH and OVX controls (Table 1.). Six animals were in each group.

Ultrasound measurements were performed 3 times: before iontophoresis, after the fifth treatment and at the end of the protocol on day 150.

3.4.5.2. Extended *in vivo* experiments on rats in order to determine the effects of topical calcium and phosphate iontophoresis versus systemic estrogen therapy (Study 3)

Twelve-week-old animals were randomly assigned into two major categories of sham-operated (n=16) and ovariectomized animals (n=24). These main groups were further divided into subgroups in a randomized manner. Within the major categories, animals in Group 1 and 2 were sham-operated or ovariectomized and were not subjected to any further interventions other than ultrasound measurements (Sham, n=10 and OVX, n=6) (Figure 4).

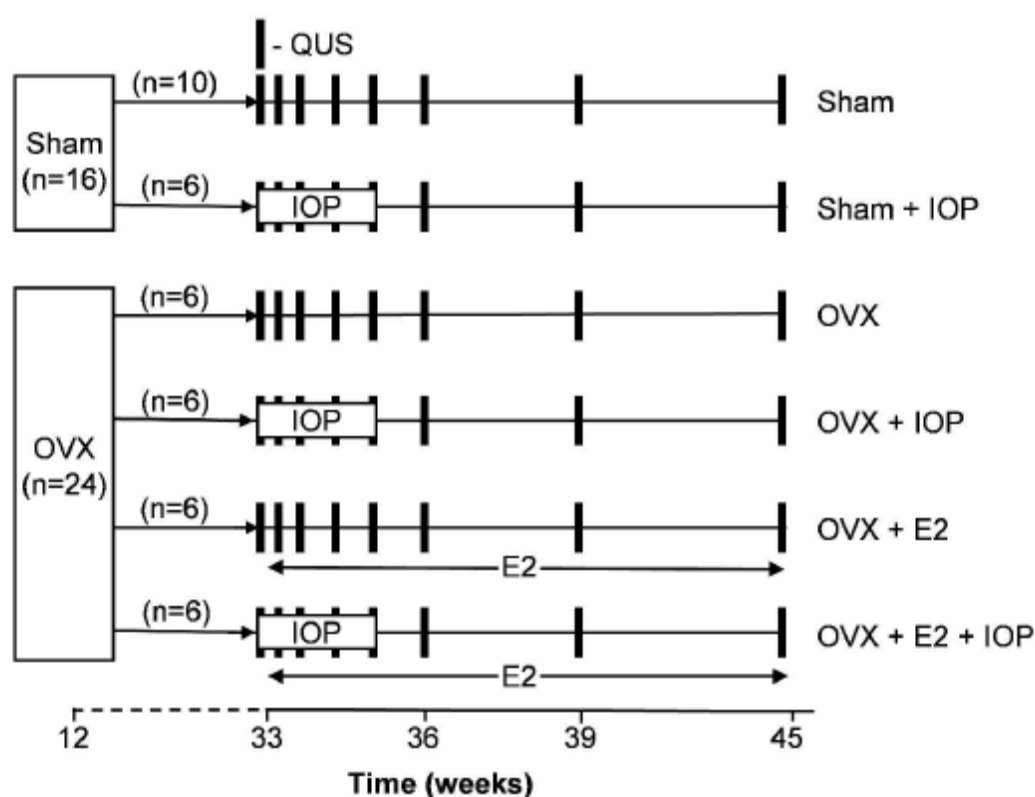


Figure 4. The time sequence of surgical interventions, treatments and measurements.

OVX = ovariectomy; Sham = sham operation; QUS = ultrasonic densitometry; IOP = calcium-phosphate iontophoresis, E2 = 17 β -estradiol treatment.

Other sham-operated and ovariectomized animals were subjected to serial local IOP treatment (Group 3, Sham+IOP, n=6 and Group 4, OVX+IOP, n=6, respectively) at the age of 33 weeks of the rats. At the same time continual E2 therapy was initiated in another subgroup of the ovariectomized animals (Group 5, OVX+E2, n=6). In some of the ovariectomized animals estrogen therapy was also combined with IOP (Group 6, OVX+E2+IOP, n=6).

Ultrasonic bone density changes were detected at the age of 33, 36, 39 and 45 weeks. At the end of the experimental protocol, the animals were over-anesthetized with a single overdose of intraperitoneally administered pentobarbital (100 mg/kg), then the tibias were ejected and subjected to further biomechanical and elemental analyses.

3.4.6. Estrogen therapy

In Study 3 in order to compare the effects of IOP with estrogen therapy, persistent 17 β -estradiol (E2) medication was initiated in further subgroups of the ovariectomized animals and was continued until the end of the studies, at the age of 45 weeks of the animals. This was performed by sc. injections of 20 μ g/kg 17 β -E2 (Sigma Aldrich; dissolved in 100% ethanol and diluted in corn oil) administered 5 days a week [170-172]. The administration was started at the same as IOP treatment (OVX+E2).

Estrogen treatment was also conducted in combination with IOP (OVX+E2+IOP). The remaining two ovariectomized subgroups (OVX and OVX+IOP) received vehicle treatment containing combination of 100% ethanol and corn oil only.

3.4.7. Biomechanical tests

At the end of the extended *in vivo* study (Study 3), *postmortem* removed left tibia of each rat was break-tested in a three-point bending procedure as described in the literature [173-178]. For this purpose, a servohydraulic instrument (Instron 4302, Instron, Hungary) was utilized.

Previously the soft tissues and the periosteum were removed from the tibia. Bones were stored immersed in Trish-buffered Ringer solution (pH 7.4) until further use.

For the biomechanical break-test, tibias were placed with their posterior side facing downwards between the edges of the instrument as the gap being 6 mm in length. The examined region contained the proximal and the mid-diaphysis tibia. The middle of this area

was pressed downwards with a speed of 1.5 mm/sec constantly until the bone was broken. Load and displacement values were continuously detected and recorded during the process. The maximal load ($F_{\max}$) expressed in Newton (N) was defined as the force resulting in breakage of the tibia. Stiffness (N/mm) was defined as tangent of the angle between the linear region of the load-displacement curve and the x-axis [174].

3.5. Statistical analysis

Statistical analyses were performed by the use of the SPSS 12.0 software package in Study 1. Data are expressed as mean $\pm$ standard error of the mean (SEM). P values <0.05 were considered statistically significant. Differences between groups were assessed by analysis of variance (ANOVA) test followed by Fisher's protected least significant difference test.

The results of the *in vivo* studies are expressed as means $\pm$ standard error of the mean (SEM). Data analyses were performed by the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA) and with the SPSS 17.0 software. Changes in AD-SoS between and within groups were analyzed by two-way repeated measures ANOVA. For other parameters, differences between groups were analyzed by two-way ANOVA. Pairwise comparisons were performed based on estimated marginal means; p values were corrected by the Bonferroni method. Differences between p values <0.05 were considered statistically significant.

4. RESULTS

4.1. Study 1: Preliminary and extended *in vitro* experiments of simultaneous calcium and phosphate IOP in porcine tissues

4.1.1. Calcium analysis in preliminary studies

When only iontophoretic current was applied without using calcium or phosphate donor microparticles, the calcium content of the muscle tissue on the anode side decreased after IOP (Group B: $608.74 \pm 8.6 \mu\text{g/g}$) compared to controls (Group A: $811.15 \pm 33.34 \mu\text{g/g}$) ($p < 0.05$). No significant differences were found between Group A and Group B in other tissues (Figure 5).

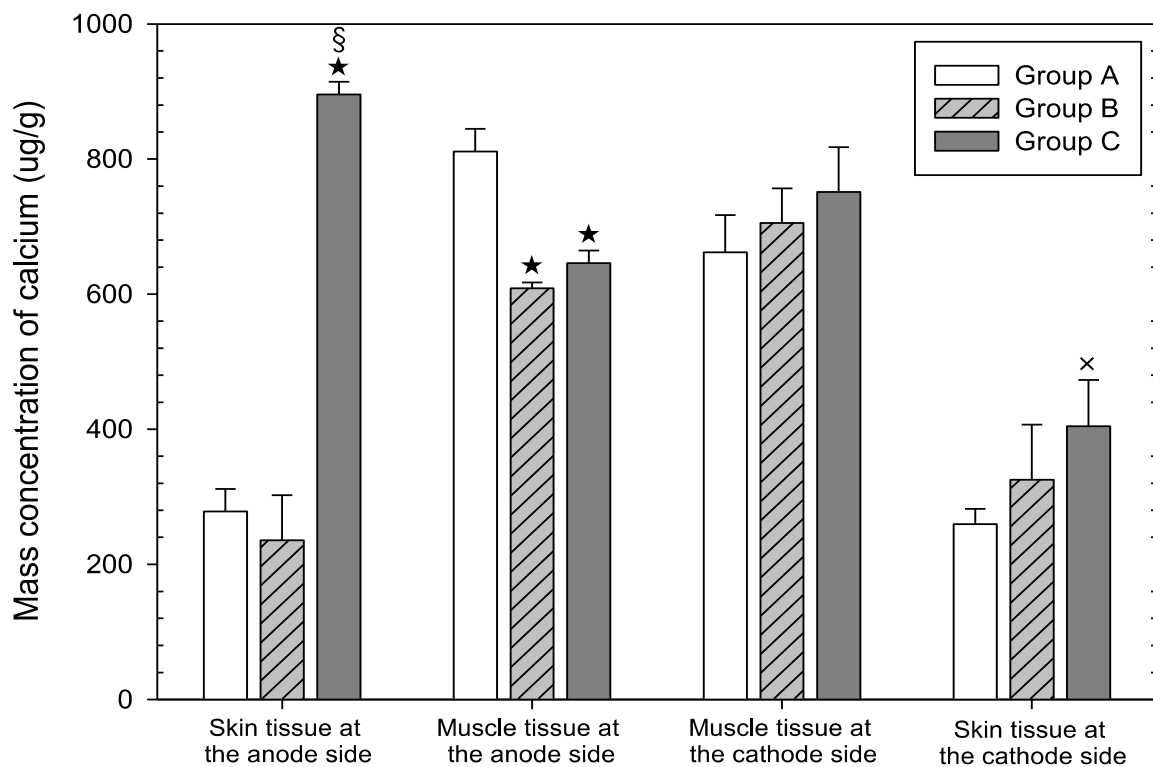


Figure 5. Calcium content of porcine soft tissues after IOP.

Group A: controls, Group B: IOP only, Group C: IOP with calcium- and phosphate-donor molecules. Values represent means $\pm$ SE ($n=6$).

★ $p < 0.05$ vs. Group A, § $p < 0.05$ vs. Group B, × $p < 0.05$ vs. Skin tissue at the anode side of Group C. Two-way ANOVA was used. Pairwise comparisons were performed based on estimated marginal means; p values were corrected by Bonferroni method

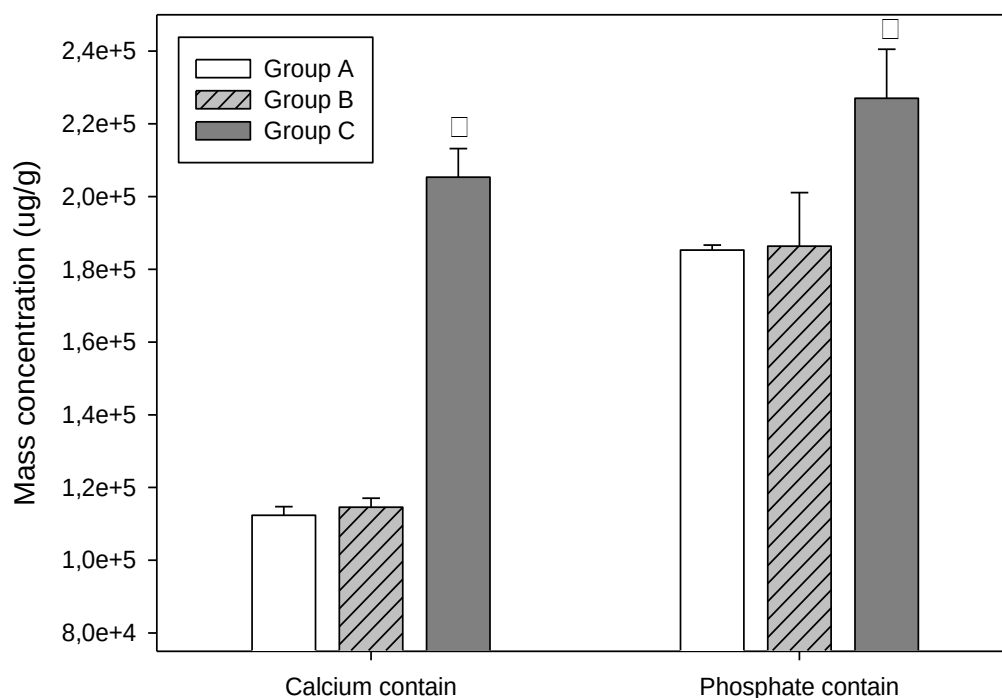


Figure 6. Calcium and phosphate content of porcine bone after IOP.

Group A: controls, Group B: IOP only, Group C: IOP with calcium- and phosphate-donor molecules. Values represent means $\pm$ SE ($n=6$). $\star p<0.05$ vs. Group A. Two-way ANOVA was used. Pairwise comparisons were performed based on estimated marginal means.

When the ion donating layers were applied, the calcium concentration of the skin and bone tissues significantly increased (Group C: $895.5 \pm 18.7 \mu\text{g/g}$ and $205334.8 \pm 7842.28 \mu\text{g/g}$, respectively) in comparison to controls (Group A: $278.26 \pm 33.19 \mu\text{g/g}$ and $112367.4 \pm 2374 \mu\text{g/g}$, respectively) ($p<0.05$) (Figure 6).

Among the different tissue samples of Group C, bone tissue contained the highest amount of calcium ($\Delta m_c = 92967.4 \mu\text{g/g}$).

4.1.2. Phosphate analysis in preliminary studies

Applying the iontophoretic current itself had no significant effects on phosphate content of the tissues as no differences were found between Group A and Group B (Figure 7).

When using calcium and phosphate donor molecules for IOP, the phosphate content of the skin significantly increased at mainly on the cathode side (Group C: anode side: $407.89 \pm 32.27 \mu\text{g/g}$; cathode side: $557.92 \pm 32.02 \mu\text{g/g}$) in comparison to negative controls (Group A: anode side: $298.61 \pm 46.66 \mu\text{g/g}$; cathode side: $329.9 \pm 92.55 \mu\text{g/g}$) ($p<0.05$) (Figure 7).

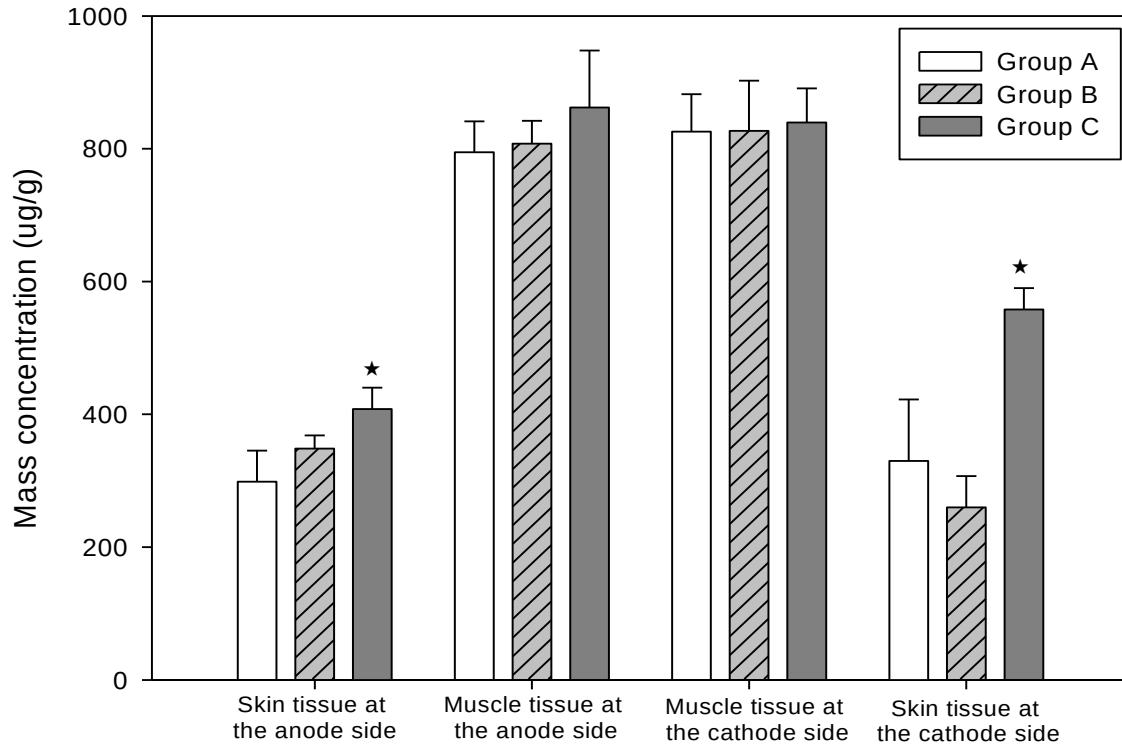


Figure 7. Phosphate content of porcine soft tissues after IOP.

Group A: controls, Group B: IOP only, Group C: IOP with calcium- and phosphate-donor molecules. Values represent means $\pm$ SE ($n=6$). * $p<0.05$ vs. Group A. Two-way ANOVA was used. Pairwise comparisons were performed based on estimated marginal means.

In comparison to controls (Group A: $185248.63 \pm 1417.33 \mu\text{g/g}$), the phosphate content of the treated bone tissue was significantly higher (Group C: $227025.33 \pm 13446.33 \mu\text{g/g}$) ($p<0.05$) (Figure 6).

4.1.3. Adequate parameters of IOP

During the experiment process no harm of the tissues was detected, although the IOP was found effective for calcium and phosphate delivery into bones (data not shown on figures). Thus the parameters utilized in this preliminary study, detailed in the ‘Method’ issue, proved to be applicable for further investigations.

4.1.4. Calcium analysis in extended studies

In our further, extended *in vitro* experiments, we wished to determine how calcium and phosphate ions are transferred to porcine tissues by the novel IOP method. When only iontophoretic current was applied without using the donor microparticles, the calcium

content of the muscle tissue on the anode side decreased after the process (Group 2: $235.7 \pm 66.7 \mu\text{g/g}$) compared to controls (Group 1: $278.2 \pm 33.2 \mu\text{g/g}$) ($p < 0.05$) (Figure 8A). No significant differences were found in other tissues (Figure 8).

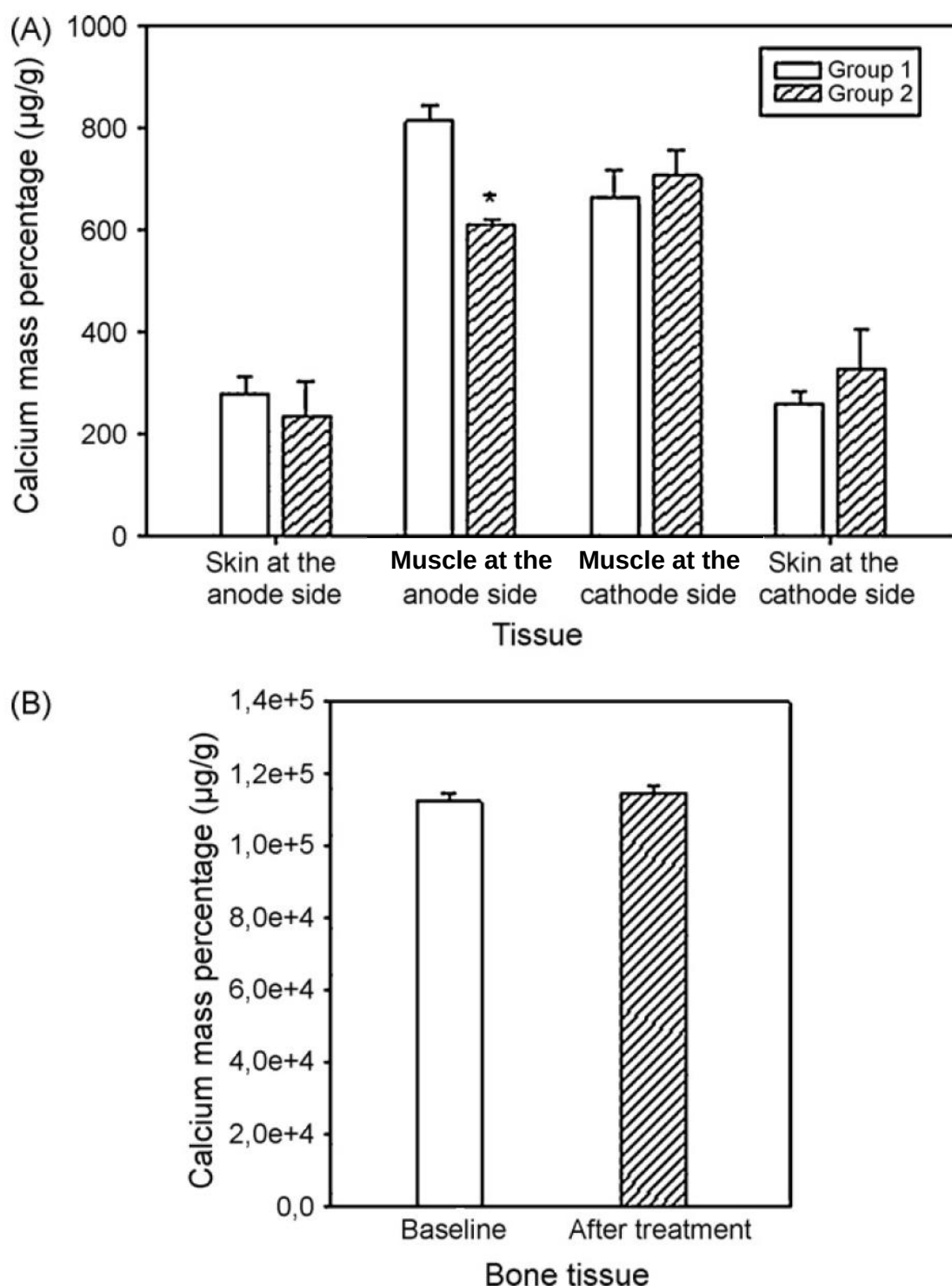


Figure 8. Calcium content in porcine tissues treated with iontophoretic current without using calcium and phosphate donor molecules.

A: skin and muscle tissues, B: bone. Group 1: controls, Group 2: IOP current treated. * $p < 0.05$, p values were corrected by Bonferroni method

When calcium and phosphate donor molecules were also applied, calcium content of the skin (Figure 9A) and bone (Figure 9B) significantly increased in the treated tissues (Group 3: $793.0 \pm 108.6 \mu\text{g/g}$ and $207628 \pm 16198 \mu\text{g/g}$, respectively) in comparison to controls (Group 1: $319.7 \pm 38.9 \mu\text{g/g}$ and $124560 \pm 15551 \mu\text{g/g}$, respectively) ($p < 0.05$).

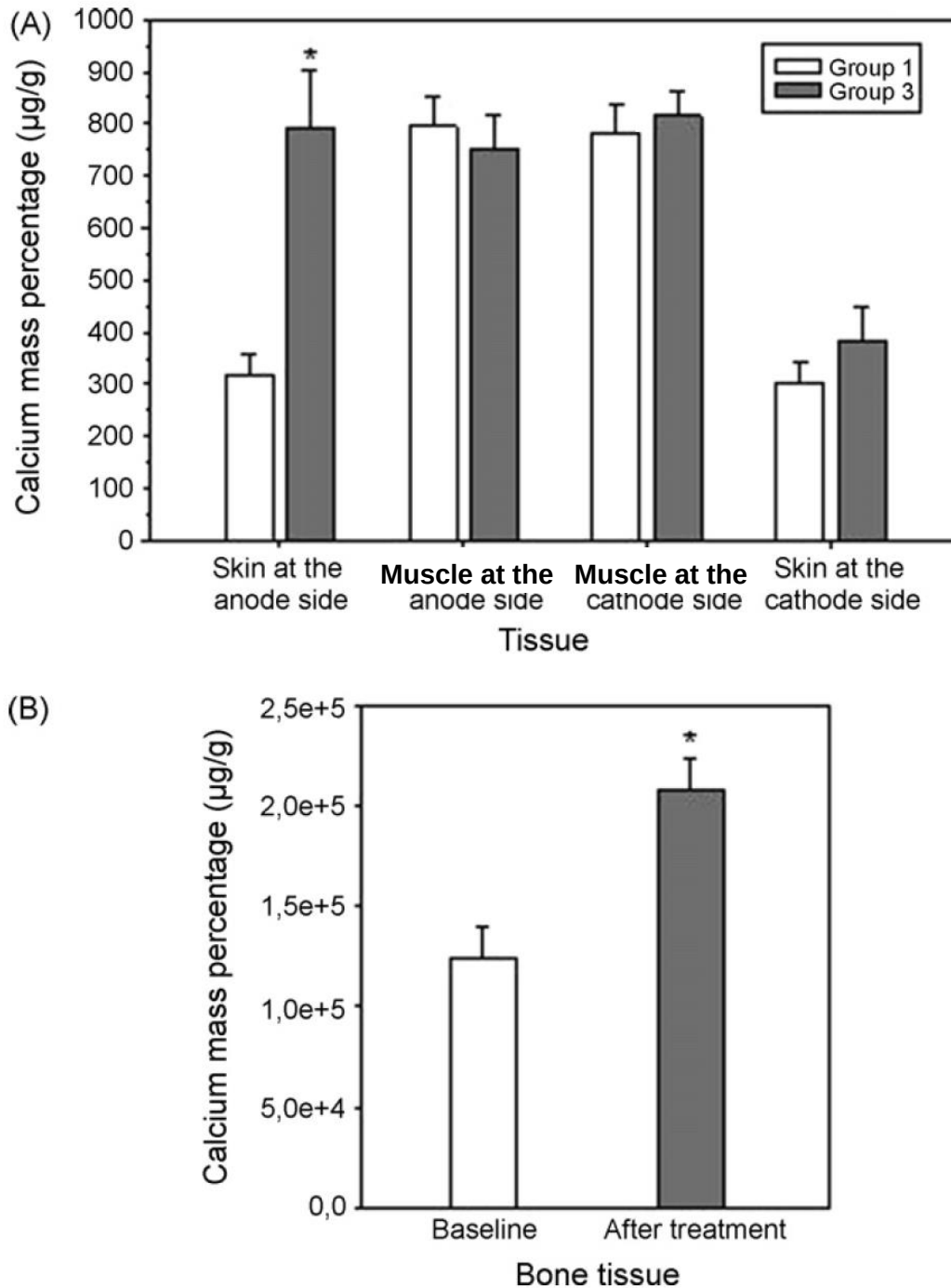


Figure 9. Calcium content in pig tissues treated with iontophoresis using calcium and phosphate donor molecules.

A: skin, muscle, B: bone. Group1: controls, Group3:IOP treated. Values represent means $\pm$ SE (n=15) $\star p < 0.05$. Pairwise comparisons were performed based on estimated marginal means; p values were corrected by Bonferroni method.

Among the samples of Group 3, the bone tissue acquired the highest amount of calcium ions ($\Delta m_c = 83068.8 \mu\text{g/g}$) (Figure 9B).

4.1.5. Phosphate analysis in extended studies

Iontophoretic current itself had no significant effect on phosphate content of the skin, muscle (Figure 10A) and bone tissues (Figure 10B) as no differences were found between Group 1 and Group 2.

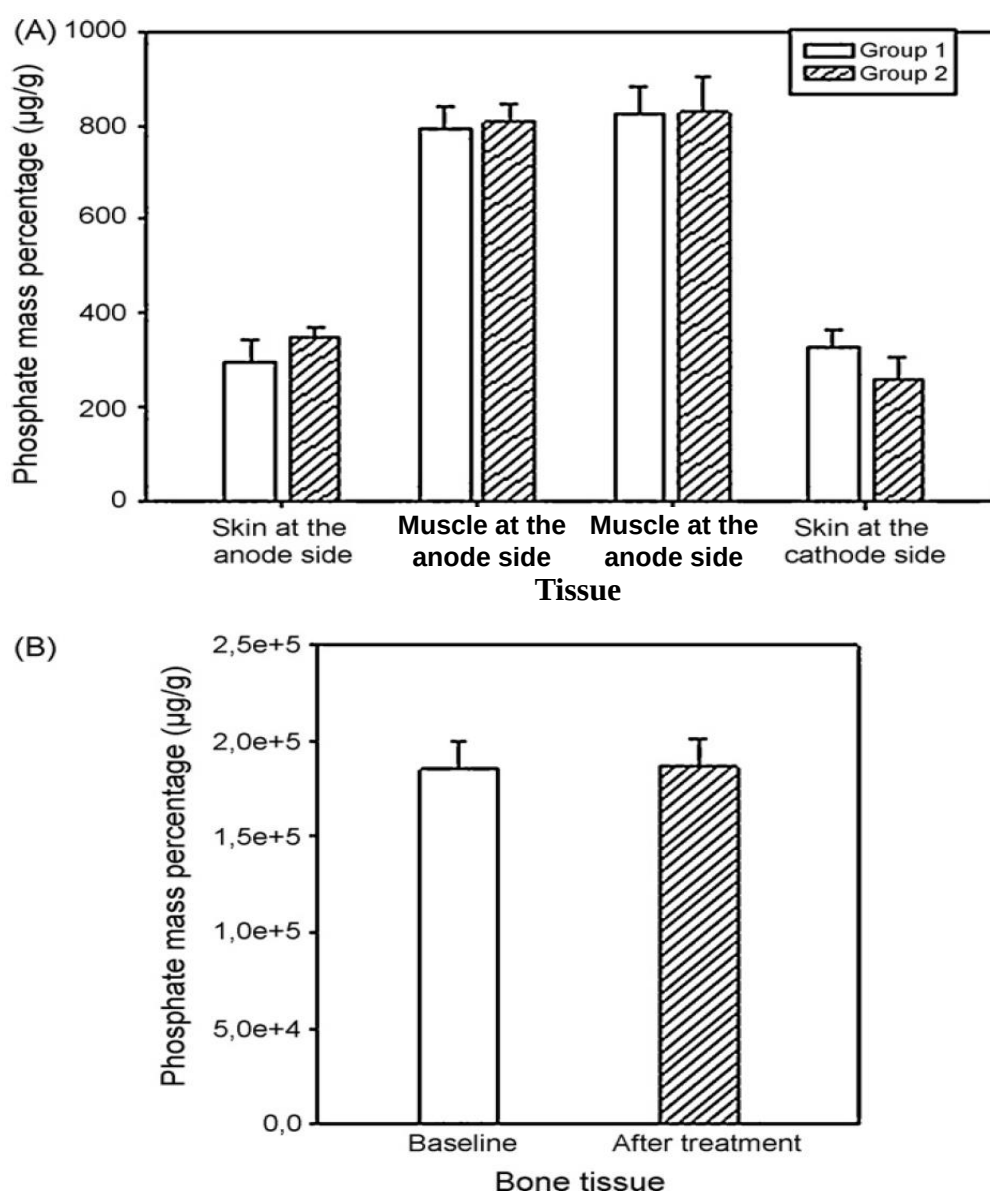


Figure 10. Phosphate content in porcine tissues treated with iontophoretic current without using calcium and phosphate donor molecules.

A: skin and muscle tissues, B: bone. Group 1: controls, Group 2: iontophoretic current. (n=15)

When using calcium and phosphate donor molecules during the iontophoretic process, the phosphate content of the skin tissue significantly increased on both sides, mainly on the cathode side (Group 3: anode side $392.7 \pm 33.2 \mu\text{g/g}$; cathode side $662.4 \pm 32.2 \mu\text{g/g}$) in comparison to untreated controls (Group 1: anode side $287.0 \pm 28.5 \mu\text{g/g}$; cathode side: $342.4 \pm 20.4 \mu\text{g/g}$) ($p < 0.05$) (Figure 11A). In comparison to controls (Group 1: $195321 \pm 10393 \mu\text{g/g}$), the phosphate content of the bone tissue was significantly higher after iontophoresis in Group 3 ($244342 \pm 13798 \mu\text{g/g}$) ($p < 0.05$) (Figure 11B).

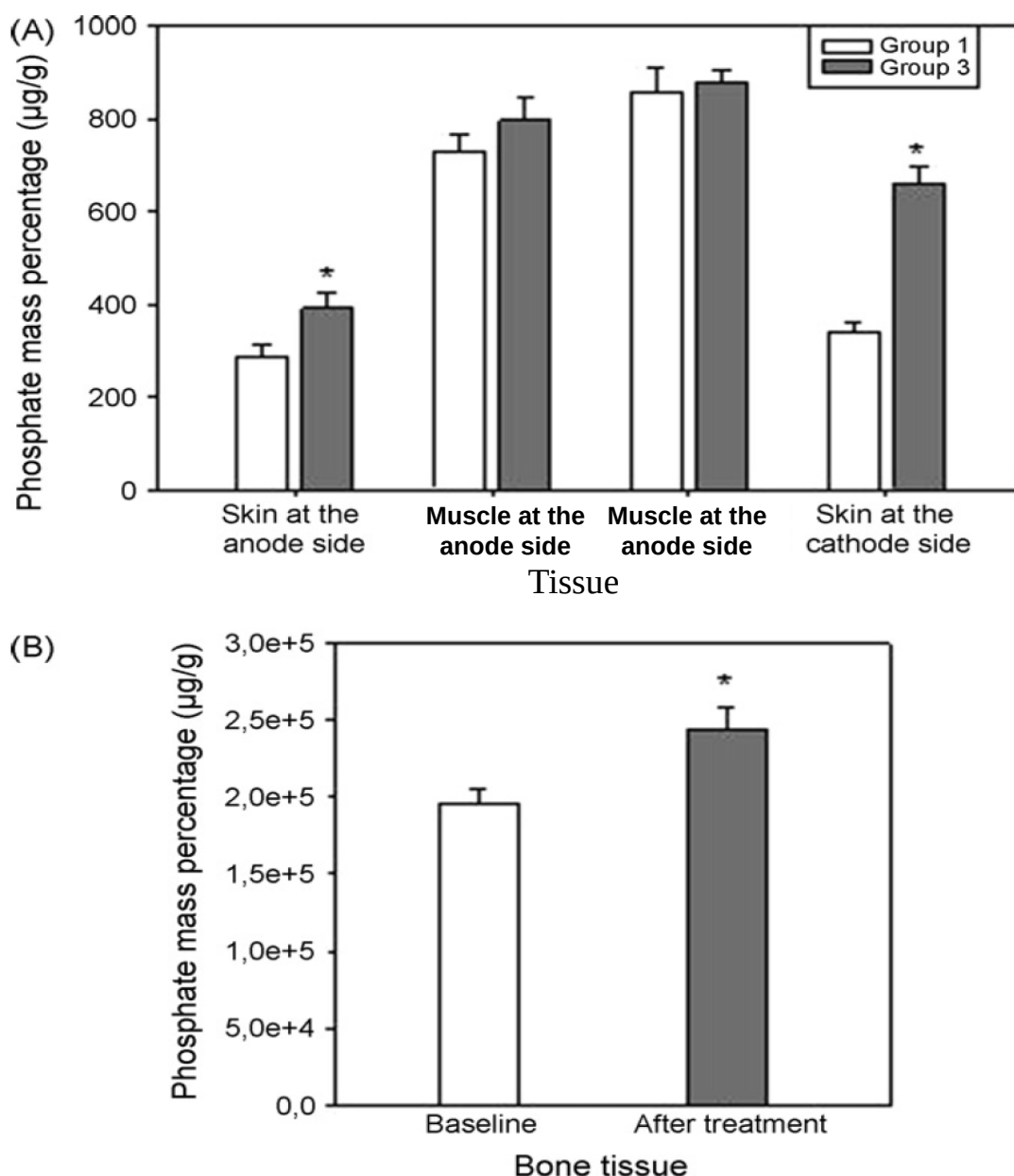


Figure 11. Phosphate content in porcine tissues treated with iontophoresis using calcium and phosphate donor molecules.

A: skin, muscle tissues, B: bone. Group 1: controls, Group 3: IOP treated with microparticles. *: $p < 0.05$ vs. control group. Values represent means $\pm$ SE ($n=15$). Pairwise comparisons were performed based on estimated marginal means.

4.2. Study 2: Preliminary *in vivo* studies on sham-operated and ovariectomized rats

As demonstrated in Figure 12, significant bone loss was detected on the tibia by quantitative ultrasound measurements in ovariectomized (OVX) rats compared to sham-operated (SH) animals.

Increased bone density was observed on treated animals as a result of five courses of IOP: significantly higher AD-SoS values were detected in both ovariectomized and sham-operated animals treated with IOP in comparison to untreated ones ($p < 0.05$).

This favorable effect of IOP on AD-SoS value was persistent even 150 days after treatment ($p < 0.05$).

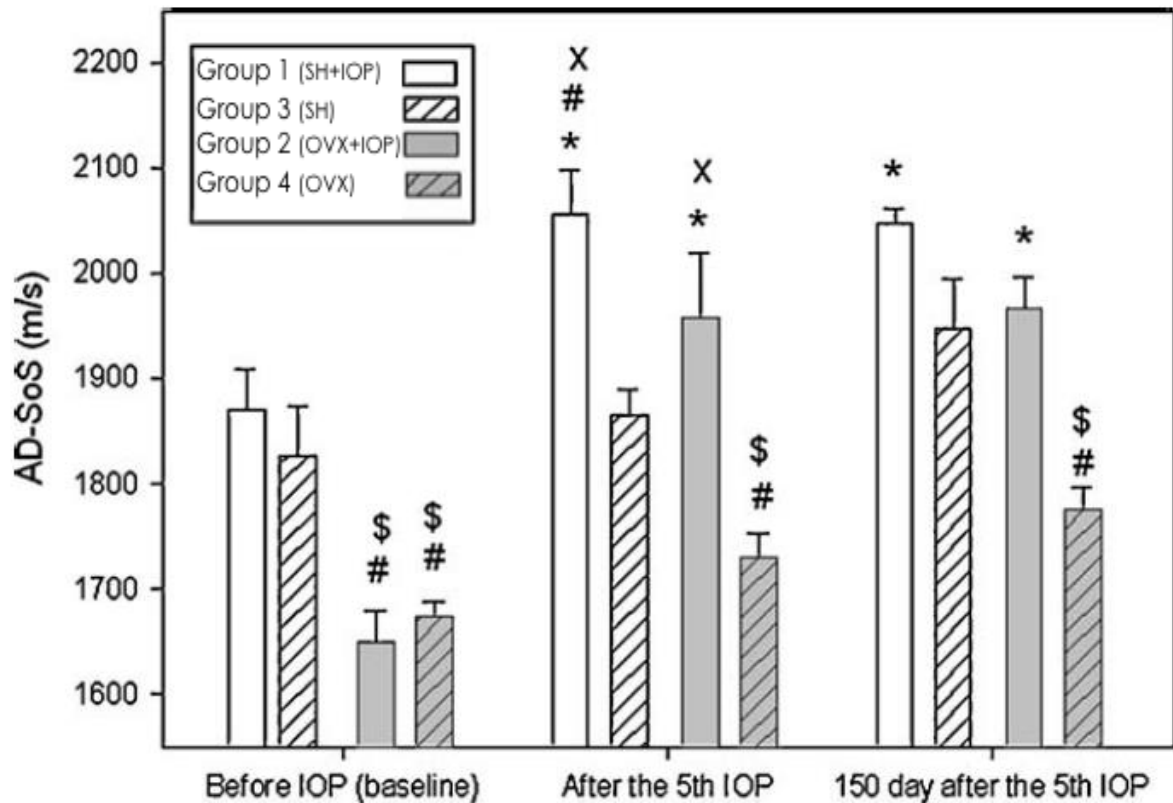


Figure 12. Effects of iontophoresis (IOP) on bone density changes in sham-operated (SH) and ovariectomized (OVX) rats.

Values represent means $\pm$ SE ($n=6$).

* $p < 0.05$ vs baseline, # $p < 0.05$ vs. SH, \$ $p < 0.05$ vs. SH+IOP, X $p < 0.05$ vs OVX;

ANOVA + Holmes Sidak test. Pairwise comparisons were performed based on estimated marginal means.

4.3. Study 3: Extended *in vivo* and *postmortem* experiments on sham-operated and ovariectomized animals in order to determine the effects of topical calcium and phosphate iontophoresis versus systemic estrogen therapy

4.3.1. Bone density changes

At the age of 33 weeks of the experimental animals, a decrease in bone density of the proximal region of tibia was detected 21 weeks after ovariectomy as shown by the AD-SoS values (Figure 13).

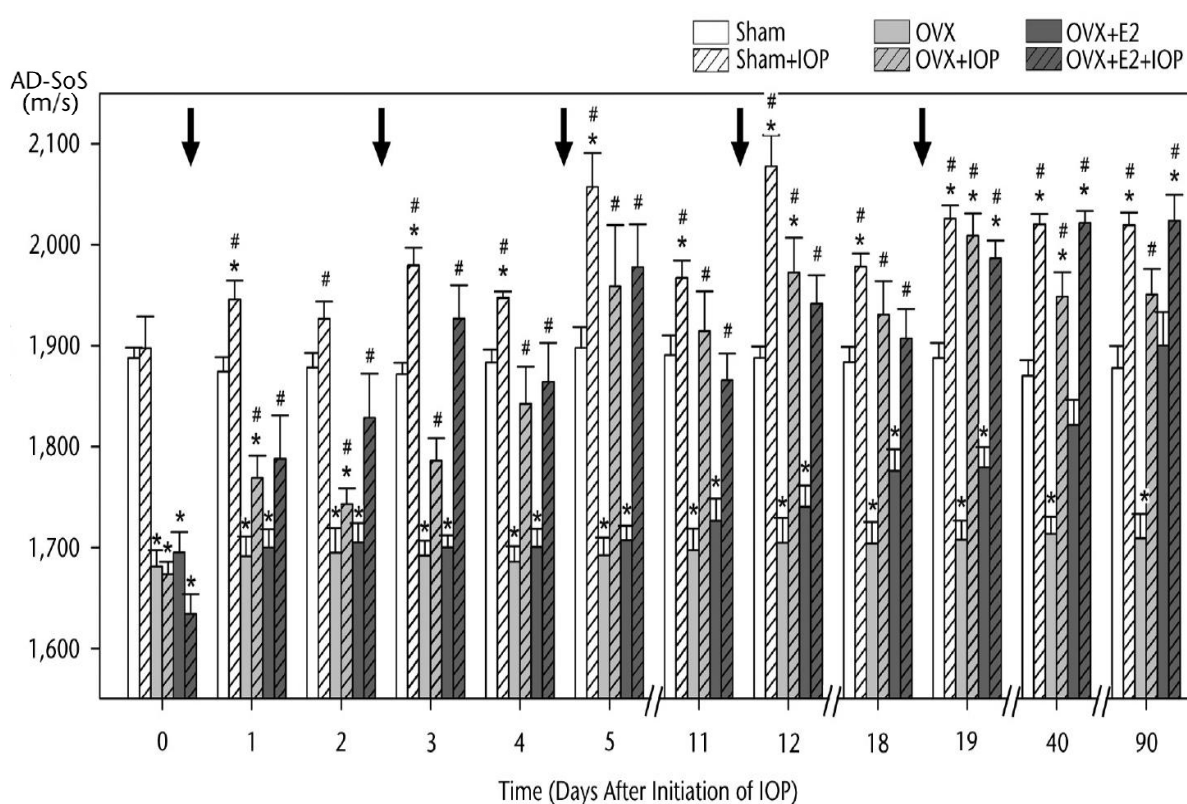


Figure 13. Time course of changes in bone density in the proximal tibia as measured by the amplitude-dependent speed of sound (AD-SoS) in sham-operated (Sham) and ovariectomized (OVX) rats and in those that were treated with topical iontophoresis (IOP) (Sham+IOP and OVX+IOP), with 17β -estradiol-treated (OVX+E2) or the combination of these treatments (OVX+E2+IOP).

Serial IOP treatments were started at the age of 33 weeks (day 0) and are marked by arrows.

Data are presented as mean $\pm$ SEM.

Two-way repeated measures ANOVA was used. Pairwise comparisons were performed based on estimated marginal means; *p* values were corrected by Bonferroni method.

* *p* < 0.05 vs Sham, # *p* < 0.05 vs corresponding group without IOP.

The differences of AD-SoS values between sham-operated (Sham) and OVX animals persisted until the end of the observation period (Figure 14). Serial IOP treatment caused sustained elevations of bone density after the third IOP treatment in all of the treated groups (Figure 14).

E2 therapy resulted in a moderate increase of bone mineral density, which reached significance by the end of the 3rd week (Figure 13). The efficacy of estrogen therapy on AD-SoS values, however, could be greatly potentiated when estrogen therapy was also combined with IOP on osteoporotic rats.

By the end of the study period, BMD was significantly lower in the OVX group than in any other group. Furthermore, IOP brought about higher density values in all of the experimental groups (Sham+IOP, OVX+IOP, OVX+E2+IOP).

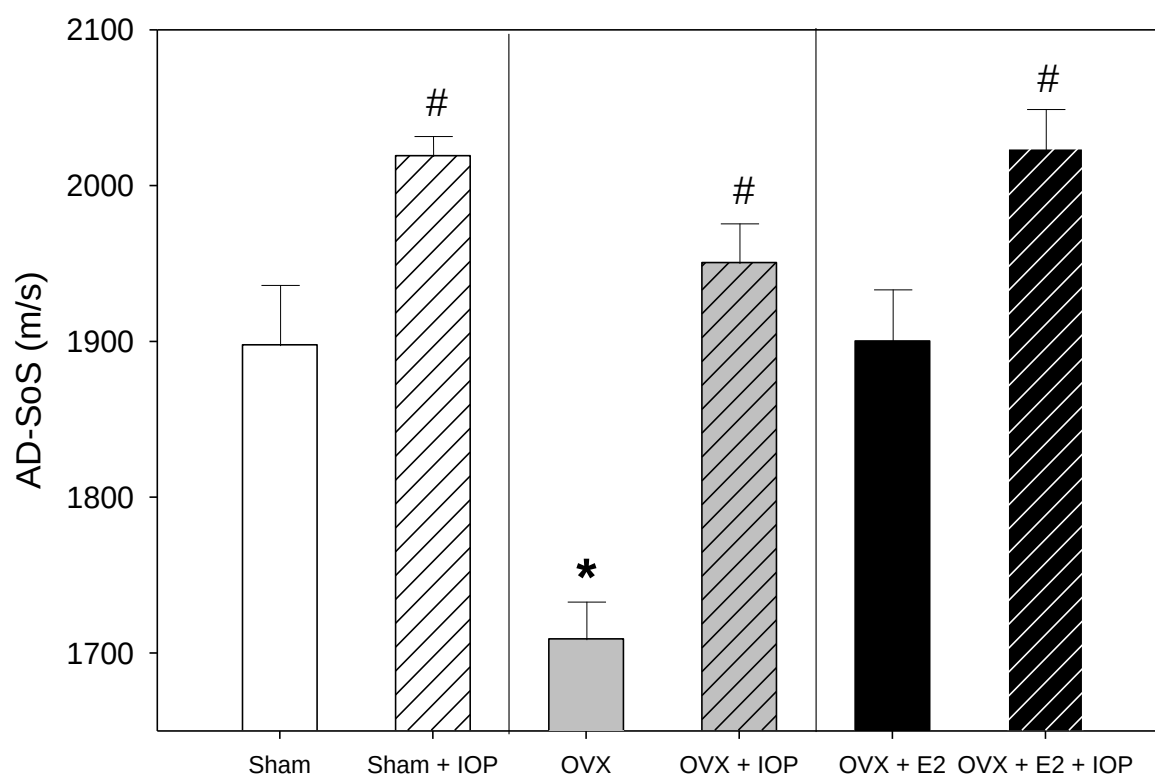


Figure 14. The effects of iontophoresis (IOP) and 17 β -estradiol treatment (E2) and their combination on the tibial bone density values measured ultrasonically (AD-SoS) in ovariectomized (OVX) and sham-operated (Sham) rats measured at the end of the experimental protocol (45 weeks of age).

Two-way ANOVA was used. Pairwise comparisons were performed based on estimated marginal means; *p* values were corrected by Bonferroni method.

* *p* < 0.05 vs OVX (between groups without IOP), # *p* < 0.05 vs corresponding group without IOP.

4.3.2. Biomechanical changes

Ovariectomy caused a significant reduction of the maximum load ($F_{\max}$) values of the tibias, but these changes were not observed if ovariectomized animals were treated with estrogen (OVX+E2) (Figure 15). As a result of IOP treatment an increase of $F_{\max}$ was observed in all groups. The value of the maximum load of the animals treated with IOP was significantly higher than the Sham and OVX+E2 animals. Likewise, $F_{\max}$ were restored to the levels of Sham animals if OVX animals were treated with IOP.

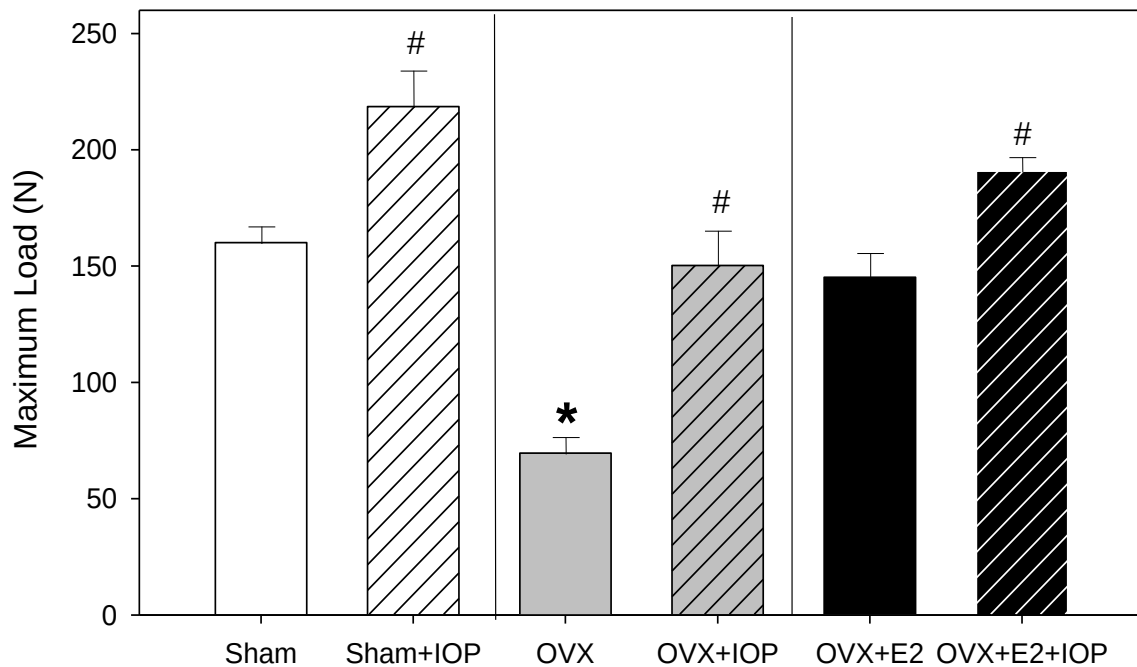


Figure 15. The effects of iontophoresis (IOP) and 17β -estradiol treatment (E2) and their combination on the maximum load of the tibias in ovariectomized (OVX) and sham-operated (Sham) rats at the end of the experimental protocol (45 weeks of age).

Two-way ANOVA was used. Pairwise comparisons were performed based on estimated marginal means; p values were corrected by Bonferroni method.

* $p < 0.05$ vs OVX (between groups without IOP), # $p < 0.05$ vs corresponding group without IOP.

Similarly, untreated OVX rats had significantly lower stiffness values than sham-operated ones (Figure 16). This loss of stiffness, however, could not be evidenced in the E2-treated OVX group. IOP caused complete restoration of this parameter in the OVX animals.

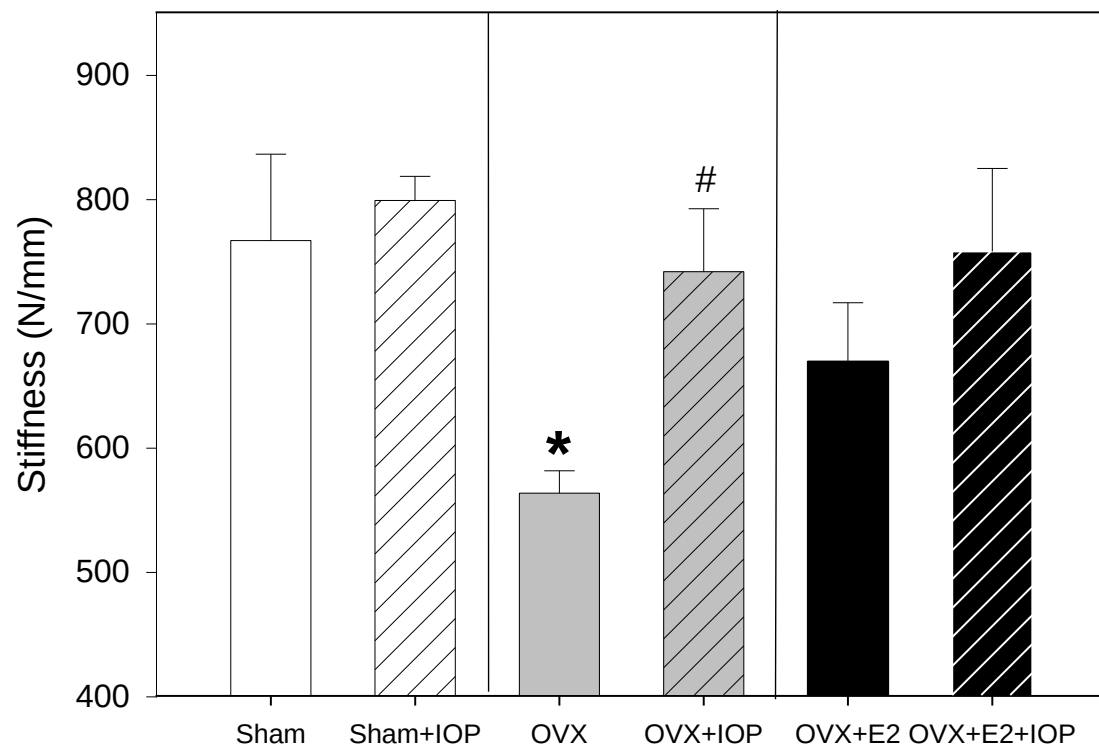


Figure 16. The effects of iontophoresis (IOP) and 17 β -estradiol treatment (E2) and their combination on the bone stiffness of the tibias in ovariectomized (OVX) and sham-operated (Sham) rats at the end of the experimental protocol (45 weeks of age).

Two-way ANOVA was used. Pairwise comparisons were performed based on estimated marginal means; p values were corrected by Bonferroni method.

* $p < 0.05$ vs OVX (between groups without IOP), # $p < 0.05$ vs corresponding group without IOP.

4.3.3. Changes in calcium and phosphate content of the bone

At the end of the study period, calcium mass concentration and concentration values were significantly lower in the ovariectomized animals than in the sham-operated group (Table 2). These measures of ovariectomy-induced bone loss were prevented by estrogen treatment (see OVX+E2). In response to IOP, calcium concentrations remained significantly elevated not only in the osteopenic OVX group, but also in sham-operated and estrogen-treated animals, even 10 weeks after the last IOP treatment.

Phosphate concentrations, however, did not show significant changes in response to ovariectomy. Calcium and phosphate IOP resulted in considerable elevations in this parameter only when Sham and OVX+E2 were combined with IOP.

Table 2. The effect of serial local iontophoresis (IOP) and 17 β -estradiol (E2) treatment or their combination on the calcium and phosphate content of rat tibias in Sham-operated (Sham) and ovariectomized (OVX) animals.

Ca mass concentration (mg/g)			
	Data (mean $\pm$ SEM)	Mean difference estimates (CI) and <i>p</i> -values vs Sham (between groups without IOP)	Mean difference estimates (CI) and <i>p</i> -values vs corresponding group without IOP
Sham	239.6 $\pm$ 1.8		
Sham + IOP	257.3 $\pm$ 1.9 [#]		17.68 (16.73-18.62) <i>p</i> < 0.01
OVX	223.8 $\pm$ 3.9 [*]	15.77 (14.83-16.72) <i>p</i> < 0.01	
OVX + IOP	234.4 $\pm$ 1.7 [#]		10.55 (9.60-11.49) <i>p</i> < 0.01
OVX + E2	233.9 $\pm$ 0.9 [*]	5.66 (4.71-6.60) <i>p</i> < 0.01	
OVX + E2 + IOP	242.9 $\pm$ 1.1 [#]		8.96 (8.01-9.91) <i>p</i> < 0.01

Ca concentration (mmol/g)			
	Data (mean $\pm$ SEM)	Mean difference estimates (CI) and <i>p</i> -values vs Sham (between groups without IOP)	Mean difference estimates (CI) and <i>p</i> -values vs corresponding group without IOP
Sham	5.97 $\pm$ 0.02		
Sham + IOP	6.42 $\pm$ 0.02 [#]		0.44 (0.42-0.46) <i>p</i> < 0.01
OVX	5.58 $\pm$ 0.04 [*]	0.39 (0.37-0.42) <i>p</i> < 0.01	
OVX + IOP	5.84 $\pm$ 0.02 [#]		0.26 (0.24-0.29) <i>p</i> < 0.01
OVX + E2	5.83 $\pm$ 0.01 [*]	0.14 (0.12-0.16) <i>p</i> < 0.01	
OVX + E2 + IOP	6.06 $\pm$ 0.01 [#]		0.22 (0.20-0.25) <i>p</i> < 0.01

PO ₄ mass concentration (mg/g)			
	Data (mean ± SEM)	Mean difference estimates (CI) and <i>p</i> -values vs Sham (between groups without IOP)	Mean difference estimates (CI) and <i>p</i> -values vs corresponding group without IOP
Sham	332.4 ± 0.4		
Sham + IOP	334.4 ± 0.9 [#]		1.93 (1.51-2.35) <i>p</i> < 0.01
OVX	331.7 ± 0.2	0.74 (0.32-1.15) <i>p</i> = 0.248	
OVX + IOP	332.4 ± 0.2		0.71 (0.30-1.13) <i>p</i> = 0.278
OVX + E2	332.3 ± 0.4	0.12 (-0.30-0.54) <i>p</i> = 1.0	
OVX + E2 + IOP	336.2 ± 0.8 [#]		3.93 (3.51-4.35) <i>p</i> < 0.01

PO ₄ concentration (mmol/g)			
	Data (mean ± SEM)	Mean difference estimates (CI) and <i>p</i> -values vs Sham (between groups without IOP)	Mean difference estimates (CI) and <i>p</i> -values vs corresponding group without IOP
Sham	3.50 ± 0.005		
Sham + IOP	3.52 ± 0.01 [#]		0.020 (0.016-0.025) <i>p</i> < 0.01
OVX	3.49 ± 0.002	0.008 (0.003-0.012) <i>p</i> = 0.248	
OVX + IOP	3.50 ± 0.002		0.008 (0.003-0.012) <i>p</i> = 0.278
OVX + E2	3.50 ± 0.004	0.001 (-0.003 -0.006) <i>p</i> = 1.0	
OVX + E2 + IOP	3.54 ± 0.01 [#]		0.041 (0.037-0.046) <i>p</i> < 0.01

Data are presented as mean ± SEM.

95% confidence intervals (CI) for mean difference are indicated in parenthesis.

Two-way ANOVA was followed by pairwise comparisons of the means.

* *p* < 0.05 vs Sham (between groups without IOP), # *p* < 0.05 vs corresponding group without IOP. *p* values are Bonferroni corrected.

4.4. Undesirable effects

Adverse reactions are classified into two groups according frequency namely “common” (>1/10) and “not known” (no occurrence in the period of the studies).

Immediately after the iontophoresis, a moderate erythema has been commonly detected (86,7%) on the skin area covered by the electrodes before. This symptom resolved spontaneously within three hours without enduring effect. Erythema of the skin was considered as the manifestation of hyperaemia.

Other skin lesions (including burn), pruritus or rash was not observed (not known). Along the whole in vivo experiments nervous system disorder (i.e. tremor, paresis, somnolence, confused behavior), cardiac disorder (i.e. syncope, circulator, collapse), respiratory disorder (i.e. dyspnoe, bronchoconstriction) or gastrointestinal symptom (i.e. diarrhea, vomiting) was not detected at any individual. Other disorders, or undesirable conditions were not under investigation.

Summary of the safety profile

After calcium and phosphate iontophoresis erythema has been occurred on the skin where the electrodes were placed before. This symptom usually resolved within a few hours. The frequency for this identified risk is common. Other moderate, mild or serious side-effect has not been observed.

5. DISCUSSION

Bone is a living tissue. It supports muscles, protects internal organs, on the other hand it serves as a mineral reservoir containing 99 percent of total calcium and 85 percent of total phosphorus of the body [1, 2, 5]. Osteoporosis results from an imbalance between bone resorption and formation in the continuous bone remodeling process. It is characterized by low bone density and a deterioration of microarchitecture that reduces bone strength leading to increased fragility and susceptibility to fractures [4].

Several systemically administered efficient pharmacotherapies are available in osteoporosis, however, local therapeutic modalities may also be useful to treat bone loss [84]. One of these techniques referred to as “calcium ionostasis” is used in the clinics as an additional therapy of Complex Regional Pain Syndrome (CRPS) and delayed callus formation [139]. Furthermore results of some studies have suggested that iontophoresis of certain compounds may have beneficial effects on fracture healing or osteoporosis [142-144].

Our research group has developed a novel iontophoretic method protected by patent that is based on the theoretical background of iontophoresis in general but differs from the techniques ever used before [149]. As a local treatment of osteoporosis, the utilization of the novel ‘three-electrode’ iontophoretic device and special calcium and phosphate donor microparticles may enable the simultaneous delivery of calcium and phosphate ions into the underlying bone. In this study, the effects and safety of the novel calcium and phosphate iontophoresis was investigated through a series of *in vitro* and *in vivo* animal experiments.

This is the first time in the literature when the targeted tissue of transdermal electrophoretic delivery is the bone. Iontophoresis with traditional Chinese herbal medicine provides a therapeutic approach for accelerating the healing of bone fractures by promoting local blood circulation [133]. The aim of calcitonin or human PTH(1-34) iontophoretic trial was to deliver the antiporotic drugs into the blood circulation [130, 131]. Magnesium ion and fluoride electrophoresis was effective in enhancing bone mineralization in teeth and maxillary bones, however it needed a direct contact of the electrodes with the treated bone tissue and the concentration of the delivered ion was not measured [128, 179]. Due to the differences of the methods, the results of my PhD work are not feasible to compare with data related to other iontophorese in literature.

In vitro experiments were carried out on porcine tissue system in order to detect the effects of iontophoretic current and calcium and phosphate iontophoresis on skin and muscle tissues as well as the transferability of calcium and phosphate ions from ion-donor molecules to the targeted tissue.

Without using calcium and phosphate donor molecules, iontophoretic current itself resulted in decreased calcium content of porcine muscle tissue close to the anode side. Although, the determined value was in the physiological range. Current itself had no effect on skin or bone tissue.

Using ion donor molecules applied to the electrodes, IOP resulted in significantly increased calcium and mildly increased phosphate content of the targeted bone tissue. Thus, results of the *in vitro* experiments suggested that calcium and phosphate ions indeed penetrated into the bone by applying IOP.

However, other tissues located above the bone are not affected by the process, except skin contacting the electrodes. The calcium and the phosphate content of the skin significantly increased as a result of iontophoresis. The enrichment of the compounds in the dermis is not surprising, as calcium- and phosphate-containing microparticles were applied on the anode and cathode, respectively, in close vicinity to the surface of the skin. The IOP had no effect on the muscles.

Results of the *in vivo* studies on ovariectomized versus sham-operated Sprague-Dawley rats, indicated that simultaneous calcium and phosphate IOP treatment increased local bone density as shown by the increase in AD-SoS values both in the sham-operated and the osteoporotic ovariectomized rats. The results suggested that the use of IOP with calcium- and phosphate-donor molecules were effective *in vivo* too without causing any harm on the experimental animals.

The positive effects could be enhanced by repeated treatment. Five times of IOP appeared to be steadily efficient, as marked by a long lasting restoration of bone density values observed even 72 days after the last treatment in all treated animal groups.

The same conclusion on effectiveness can be drawn from the results of the biomechanical tests. Bilateral ovariectomy resulted in significantly decreased maximum load and bone stiffness in comparison to the sham-operated ones. Data indicated that the novel IOP treatment had a positive effect on the biomechanical parameters too as maximum load and stiffness values of the treated tibias increased by iontophoresis.

Elemental analysis provides further support for the findings described above. Ovariectomy caused a significant decrease in calcium mass concentration, but only a moderate reduction in the phosphate content. Again, IOP increased the detected ion concentrations not only in ovariectomized rat tibias, but also in sham-operated animals.

The *in vivo* animal studies presented evidence for the efficacy of calcium and phosphate IOP concerning bone mineral contents, AD-SoS values and improved biomechanical properties of osteoporotic rat tibias.

We compared the effects of IOP as well as the effect of estrogen therapy and the combination of electrotherapy and estrogen medication on osteoporotic rats.

Bone loss and the consequences induced by ovariectomy could also be effectively reversed by chronic estrogen replacement therapy. The data also show that IOP and estrogen monotherapy appeared to be similarly effective.

The combination of the two modalities provides the highest efficacy on BMD. IOP or estrogen therapy was able to restore maximum load and stiffness lost after ovariectomy in the same extent. Likewise, the combination of the two therapeutic modalities was even more effective in normalizing the biomechanical properties of the treated osteoporotic rat tibias.

Based on the results of the calcium and phosphate elemental analyses, estrogen therapy appeared to be less effective than the combination of hormone- and electrotherapy in restoring bone mineral content.

In conclusion, by means of this local, novel three-electrode iontophoretic apparatus and the use of calcium- and phosphate-donor microparticles, bone mineral density, calcium and phosphate content and biomechanical properties of the osteoporotic bone can be effectively improved. The efficacy of this approach is comparable to that of chronic estrogen therapy and the combination of these approaches further improves the effectiveness. Hence topical iontophoresis may represent an effective novel treatment modality in the management of the local forms of bone loss.

The possible side-effects of calcium and phosphate iontophoresis are the same as of other iontophoretic treatment. Serious or mild adverse event has not been observed. Immediately after calcium and phosphate iontophoresis, erythema has been occurred on the surface of the skin where the electrodes (either anode, cathode or zero electrode) were placed before. This symptom usually resolved within 3 hours without any intervention. The

frequency of this identified risk was common. Other moderate side-effect has not been detected. Along the whole in vivo experiments the examined nervous system, cardiac and respiratory disorder or gastrointestinal symptom has not been observed at any individual.

Limitation of this novel iontophoretic method emerges mainly from its topical applicability. Therefore, this technique may be restricted to areas where the electrodes can have a relatively close proximity to the targeted bone and to each other. Potential species-dependence of efficacy and limitations derived from the depth of penetration provided by the applied method would also restrict extrapolation of the results to humans. Treatment of the most affected areas of bone loss i.e. the vertebrae may require further technical modifications. The presented experimental model also has limitations. This comprises the relatively low number of experimental animals included in these studies, and the limited clinical relevance of estrogen supplementation as a basis of comparison for the effects of iontophoresis in osteoporotic women.

For the above reasons, this novel approach may have therapeutic potential at local forms of osteoporosis such as algoneurodystrophy.

6. SUMMARY

In my Ph.D. study I aimed to perform *in vitro*, *in vivo* and *postmortem* animal experiments first in the world for the setting of a novel simultaneous calcium and phosphate iontophoretic treatment and for the detection and clarification of its effects on bone.

The new results are summarized below:

- First in the literature, both calcium and phosphate ions were simultaneously delivered transdermally into porcine bone by calcium and phosphate iontophoresis *in vitro*.
- It was proved, first time in literature, that calcium and phosphate iontophoresis has *in vivo* long-lasting effect on osteoporotic and normal rat tibias regarding AD-SoS values, biomechanical properties and mineral content improvement.
- A series of simultaneous calcium and phosphate iontophoresis compare to chronic estrogen therapy found to be similarly effective on the treatment of osteoporotic rat tibia, which effect can be enhanced by combining the two modalities.

These studies presented evidence for the efficacy of calcium and phosphate iontophoresis on bone mineral contents, AD-SoS values and biomechanical properties of osteoporotic bones. The results suggest that topical iontophoresis may be suitable for further *in vivo* utilization and probably for human use.

The limitation of the treatment derives mainly from its local applicability. Species-dependency and the experimental models used in these studies would also restrict extrapolation of the results to humans. For these reasons, the novel method may mainly have therapeutic potential at local forms of osteoporosis.

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8. KEYWORDS AND LIST OF ABBREVIATIONS

Keywords: osteoporosis, calcium, phosphate, iontophoresis, physiotherapy, rat, tibia

Kulcsszavak: osteoporózis, kalcium, foszfát, iontoforézis, fizioterápia, patkány, tibia

AAS	atomic absorption spectroscopy
AD-SoS	amplitude-dependent speed of sound
ANOVA	analysis of variance
BMC	bone mineral content
BMD	bone mineral density
BMP	bone morphogenic protein
BSAP	bone-specific alkaline phosphatase
CH	channel
CI	confidence interval
CP	courts period
CPU	Central Processing Unit
CRPS	complex regional pain syndrome
CTX	carboxy-terminal collagen crosslinks
DAC	digital-to-analog converter
DC	direct galvanic current
DEXA	dual-energy X-ray absorptiometry
DP	diphase
DPYD	deoxypyridinoline
E2	17-beta-estradiol
ECM	extracellular matrix
ESR	erythrocyte sedimentation rate
F_{max}	maximal load
HRT	hormone replacement therapy
IGF-I	insulin-like growth factor 1
IOP	iontophoresis
MP	monophase
NTX	N-terminal telopeptide of collagen type-I
OPG	osteoprotegerin

OVX	ovariectomized
PICP	procollagen I carboxyl-terminal peptide
PINP	procollagen I amino-terminal peptide
PTH	parathyroid hormone
PYD	pyridinoline
qCT	quantitative computed tomography
QUS	quantitative ultrasound bone densitometry
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
RSD	reflex sympathetic dystrophy
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
SH	sham-operated
Sham	sham-operated
sTSH	supersensitive thyroid stimulating hormone
TGF-β	transforming growth factor β
US	ultrasound
UV-Vis	ultraviolet–visible

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10. PUBLICATIONS

10.1. *In extenso publications related to the dissertation*

1. **Gomez, I.**, Szabó, A., Pap, L. Jr., Pap, L., Boda, K., Szekanecz, Z.: In vivo calcium and phosphate iontophoresis for the topical treatment of osteoporosis. *Phys Ther.* 92(2), 289-297, 2012.

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10.2. *Other publications*

3. Juhász, P., Mester, Á., Bíró, J., Héjj, G., Apáthy, Á., Bálint, P., Bazsó, A., Donáth, J., Gaál, R., **Gomez, I.**, Hittner, Gy., Hodinka, L., Hunka, A., Király, M., Kiss, Cs., Kiss, E., Korda, J., Márkus, I., Mikó, I., Orbán, I., Ormos, G., Ortutay, J., Penczner, G., Polgár, A., Ruzicska, É., Schmidt, Zs., Seszták, M., Sevcic, K., Sterba, G., Vereckei, E., Winkler, V., Poór, Gy.: A biológiai és a hagyományos betegségmódosító terápia hatékonyságának összehasonlító vizsgálata magyarországi reumatoid arthritisek betegekben: az ABRAB-vizsgálat első eredményei. *Magyar Reumatol.* 53(2), 63-73, 2012.
4. **Gomez, I.**, Nagy, D., Seszták, M., Vereckei, E., Csauth, K., Farkas, P., Hodinka, L.: Szeptikus arthritisek diagnosztikai kihívásai két eset kapcsán. *Magyar Reumatol.* 52(4), 207-212, 2011.
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10.3. Abstracts

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10.4. Referatum

22. **Gomez I:** Gyógyszeres terápia a csonttörés kockázatának csökkentésére: Észrevételek az ACR clinical practice guideline-jaival kapcsolatban. Immunol. Szle. 1(3-4), 42, 2009.
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