The modern approach of the post-menopause osteoporosis etiology relies on the unbalance between the processes of bone resorption and formation, caused by the lack of estrogen hormones which determine different combined forms of increasing/decreasing the osseous cell activity, osteoblasts and osteoclasts by type. With regards to this, some histomorphometric analyses are carried out in order to determine the mineralized bone and osteoid relative quantity, and also the activity of cells having a formation function (osteoblasts) or a resorption of the osseous tissue (osteoclasts).

American Society for Bone and Mineral Research recommend the evaluation of the following histomorphometric parameters: the volume of the osseous trabeculae (BV/TV, % - the percentage of the osseous tissue in a certain volume. Schematically, it represents the report full/empty within the bone); osteoid area (OS/BS, % - the percentage of the surface of the bone formed on a certain bone area); osteoblast area (Ob. S/BS, % - the percentage of the spongy bone trabecular and span areas with morphologically-active osteoblasts); osteoid volume (OV/BV, % - the osteoid percentage within a certain bone volume); osteoid width (O. Th, m, - the average width of the osteoid tape, which represents the support for calcification); osteoclast area (Oc. S/BS, % - the percentage of the trabecular areas with resorption gaps occupied by one or many osteoclasts, considering the fact that calcium deficiency causes the increase of osteoclast number and stimulates their activity); mineralization rate through apposition (MAR, m/day), calculated in divisions per average distance between two fluorescent tagging (it results from calceine accumulation at the mineralization front level). This double tagging has been accomplished through calceine i.m. injection at intervals of 6 days, 48 hours before sample taking. The researches presented in this paper work belong to the subcontract CEEX no. 110-2, partner no. 2, within the contract CEEX 110 entitled "Experimental model for the study upon the bioavailability of some nutritional factors (Ca, B, phytosterols) and their influence upon bone mineralization in pigs, a scientific support in the study upon osteoporosis”.

Key words: osteoporosis, mineralization, osteoblasts, osteoclasts, osteoid, histomorphometry
The osseous tissue is consisted of: osseous cells (osteoblasts – young cells; osteocytes – adult cells; osteoclasts) and an osseous matrix consisted of fundamental substance (osseine), impregnated with hydroxyapatite crystals ($\text{Ca}_{10}$$\text{(PO}_4\text{)}_6\text{(OH)}_2$) and conjunctive fibers organized in blades.

Osteoblasts, also name the bone line cells (BLCs), are the cells which form the bone. They generate from osteoprogenitor cells (mesenchymal cells), localized near bone surface (within the internal periosteum layer) and within the cavities housing the bone marrow (medullar channel, respectively the spongy bone areoles), where they differentiate under the influence of the growth factors (e.g. the fibroblastic growth factor – FGF, the growth factor derived from the sanguine platelets – PDGF, the transforming growth factor beta-TGF-$\beta$ and other proteins with a role in bone morphogenesis – BMPs). The activity of alkaline phosphatase (ALP) occurs intensely within the osteoblast cytoplasm, this enzyme playing an important role in bone mineralization.

Osteoblasts exert a rich photosynthesis activity, being actively involved in the synthesis of proteins and of the fundamental substance mucopolisaccharides (osseine). So, the osteoblast releases the osteoid, consisted especially of collagen molecules type I and of non-collagenic proteic molecules, like osteonectine, osseous proteoglycans and bone sialoprotein.

Such a non-collagenic protein is represented by the osteocalcine, also called the bone gla-protein (BGP), whose synthesis depends upon the vitamin K. It represents the index specific for the osteoblast activity and reflects directly the bone turnover.

In human species, the osteoblast produces an about 15 $\mu$m wide osteoid layer, with a deposition degree of about 0.5-1.5 $\mu$m per day (Jowsey et al., 1977). Osteoblasts also release a series of hormones and prostaglandins too, which act upon the bone itself and enzymes (e.g. ALP, ATP-ase, hydrolase). Alkaline phosphatase (ALP) facilitates the mineralization of the osteoid matrix, representing the phenotypical marker for osteoblasts. Within the matrix formed, some mineral salts (phosphates and calcium carbonates) will depose as hydroxyapatite crystals ($\text{Ca}_{10}$$\text{(PO}_4\text{)}_6\text{(OH)}_2$) – generating mineral deposition nuclei surrounding the osteoblasts, which remain included in the new-formed tissue gaps.

The osteoclast is a multi-nucleate cell which involves in bone degeneration and resorption. It is involved in the natural turnover of the osseous tissue together with the osteoblasts. Osteoclasts are provided by the stem cells of the monocitary line (CFU-GM), and belong to the body monocitary-macrophage system. Osteoclast role is to remove the excess of osseous tissue, especially during osteogenesis periods. Osteoclast activity is regulated by different hormones, including the parathormone (PTH) released by parathyroid, the calcitonine released by the C cells from the thyroid gland and the growth factor interleukin 6 (IL-6).
This last factor IL-6, is one of the factors involved into osteoporosis, a disease caused after unbalance between the processes of bone resorption and formation.

The bone matrix is consisted of organic and inorganic matter, in equal proportions. The organic substance contains 95% type I collagen fibers (consisted of two chains $\alpha_1$ and one chain $\alpha_2$) and glycosaminoglicans. The collagen is stored initially as osteoid (the new formed bone matrix, before calcification) (Rubin and Sledge, 1993). Within the skeleton of a normal adult, the collagen is consisted of dense layers of about 3 $\mu$m width on the surface of the preexistent bone, immediately adjacent to marrow.

The matrix also contains a series of non-collagenic proteins. This category includes osteocalcine (bone $\gamma$-carboxyglutamic acid, BGP or the bone gla-protein), representing up to 12% of the vertebrate bone protein. The synthesis of this protein is emphasized after osteoblast stimulation with vitamin D$_3$ (de Brum-Fernandes et al., 1994).

Among the non-collagenic proteins (glycoproteins and phosphoproteins), we should also mention osteonectine (Fischer et al., 1987), osteopontine (Reinholt et al., 1990, Noda et al., 1988), sialoprotein (Vaananen, 1993), a small number of proteoglycans, etc. (Skerry et al., 1990).

**Material and methods**

Most histomorphometric techniques must be applied on non-decalcificated sections. In order to assess the mineralization rate through apposition, before sample taking we should treat the individuals with tetracycline (about 10-15 mg/kg body weight). Bone samples, taken with the width of 3-5 mm, are fixed in neuter formalin 10% or ethylic alcohol 70°, dehydrated in ethylic alcohol solutions with increasing concentrations (70°, 96°, 100°, about 1 hour/bath), and then included into a resin-based medium. On the whole, the acrylic resins are used more frequently (e.g. methyl metacrylate), because they assure a much detailed cell morphological superiority. The metacrylate is initially used as monomer, and then the samples will be introduced into a solution of partially polymerized methyl metacrylate, which will polymerize at the temperature of 37°C for a few days.

In the immune-histochemical and histoenzymatic techniques, due to the low temperatures necessary for polymerization, the medium K-Plast is recommended – an inclusion metacrylate-based medium, with adhesive features. In this case, sample fixation may be carried out in neuter formalin 10%, at 4°C, for 4-16 hours. Block sectioning may be carried out at 5 $\mu$m.

Successive to section adhesion onto the plate, the resin is removed by immersing the plates into acetone for about 30 minutes, and then the coloration will be accomplished depending on the objective aimed.

Section coloring may be carried out using the following methods: von Kossa, Van Gieson (the variant Unna), the trichromic Goldner, with toluidine blue. All these methods highlight the places in which calcium depose, the osteoid, the bone cell and fiber formations.
The degree of bone osteoclastic resorption may be visualized by highlighting the acid phosphatase resistant to tartaric acid (TRAcP).

The verification of some enzymes activity (e.h. alkaline phosphatase – marker for bone mineralization; acid phosphatase – marker for osteoclast resorption activity) may be carried out on tissue decalcificated in EDTA, and the estimation of the bone volume in section may be carried out with acids. Bone structural parameters may be also estimated on decalcificated sections, being easy to differentiate them by the other non-bone components.

The measuring of the bone tissue surface and, through extrapolation, of the relative volume, and also the estimation of a bone structure width are possible with direct morphometric methods.

Depending on the investigation complexity, the equipment used in histomorphometry ranges from a simple ocular micrometer to very sophisticated computer-controlled apparatus, with image analysis systems.

**Results and discussions**

Pig’s rapid rate of growth and its high level requirements for mineral substances allow this species to be used as model-animal for the study upon calcium requirements in human species. So, it is known that, in human species, the calcium requirement ranges from 600 mg for children to 900 mg/day for adults, and in pigs, the calcium requirement ranges from 7 g/day for 10 kg body weight to 21 g/day at the weight of 70 kg (Gueguen and Pointillart, 1995, 1986, quoted by Eklou-Kalonji, E et al., 1997). This “avidity” for calcium is one of the reasons why the pig is a sensible model for calcium quantity and amount level, leading to a series of experimental researches with regards to calcium availability in human species (Pointillart and Guegues, 1993, Pointillart et al., 1995, quoted by Eklou-Kalonji, E. et al, 1997) and at the same time to the determination of a series of parameters related to bone metabolism: biochemical (bone metabolism markers), mechanical and histological, for the evaluation of calcium deficiencies’ effects at bone level.

Calcium plays an important role in bone physiology and homeostasis. It is stored within bone during its formation and is released during bone resorption. Calcium serum concentration represents the major factor which regulates the process of bone re-modeling. By using some calcium-deficient diets, we may investigate hypocalcemia’s effect upon bone development. On the whole, the main hypocalcaemia-based changes are consisted of: the reduction of bone formation rate; bone loss or the osteoporosis (Sisson, H.A. et al., 1984; Ohya, K., 1994; Shen, V. et al, 1995); the increase of osteoclast number and the intensification of the bone resorption process or the osteoclasis (Liu, C.C. et al., 1982, Ohya, K., 1994); the increase of endosteal cell number, and rachitis and osteomalacia in young animals (Pettifor, J.M. et al., 1984).

Recent researches reveal boron implication in the synthesis of estrogens, vitamin D and of other steroid hormones, being essential in the process of adding
the group -OH to hormone molecules, respectively vitamin D. The presence of the
–OH groups determines a great difference in the hormonal characteristics, the
differences between testosterone and estrogen being especially caused by a single
hydroxyl group (-OH).

The histomorphometric analyses carried out on bone samples taken from
piglets which received in their forage different calcium amounts prove that the
volume of the bone trabeculaes was significantly diminished (p<0.01) in the groups
which did not receive calcium, compared to the control group. Osteoid width has
increased remarkably (x2.5) at the same time with the degree of increasing calcium
deficiency (through the lack of osteoid mineralization).

In all mineral-deficient animals (especially calcium) the osteoclasia is
strongly stimulated, leading to a significant increase of the osteoclastic surfaces in
animals which did not receive calcium, compared to the control variant, while the
osteoblastic surfaces has increased only in the high-deficient animals (0%
calcium).

So, in spite of the hypocalcaemia absence, the stimulation of bone
resorption (the increase of osteoclastic surfaces) has occurred already in the
medium calcium-deficient animals, and in exchange, the process of bone formation
(the increase of osteoblastic surfaces) has been stimulated only in the animals that
were submitted to a remarkable hypocalcaemia.

Bone mineralization speed is strongly diminished in the calcium-deficient
animals, as much as the deficiency degree increases, compared to the control
group.

**Conclusions**

1. Histomorphometric analyses are used in order to determine the relative
quantity of mineralized bone and of osteoid and also the activity of cells with a
forming function (osteoblasts) or function of bone tissue resorption (osteoclasts).

2. American Society for Bone and Mineral research recommend the
evaluation of the following histomorphometric parameters: the volume of the
osseous trabeculaes (BV/TV, %); osteoid surface (OS/BS, %); osteoblasts surface
(Ob. S/BS, %); osteoid volume (OV/BV, %); osteoid width (O. Th, m,); osteoclasts
surface (Oc. S/BS, %); mineralization rate through apposition (MAR, m/day).

3. Most histomorphometric techniques must be carried out on non-
decalcificated sections.

4. Section coloration may be done with the following methods: von Kossa,
Van Gieson (the variant Unna), trichromic Goldner, with toluidine blue.

5. The measuring of the bone tissue surface and, through extrapolation, of
the relative volume, and also the estimation of a bone structure width are possible
with direct morphometric methods.

6. Pig is used as model-animal for the study upon calcium requirements in
human species due to its rapid growth speed and to its big requirements for mineral
substances, similar to the human species.
7. Calcium adsorption at intestinal level is a complicated process, which involves sexual hormones presence, especially estrogens.

8. Recent researches reveal boron implication in the synthesis of estrogens, vitamin D and of other steroid hormones, being essential in the process of adding the group -OH to hormone molecules, respectively vitamin D.

9. In all mineral-deficient animals (especially calcium) the osteoclasts is strongly stimulated, leading to a significant increase of the osteoclastic surfaces in animals which did not receive calcium, compared to the control variant, while the osteoblastic surfaces has increased only in the high-deficient animals (0% calcium). The process is accompanied by the decrease of the bone trabeculae volume.

10. Osteoid width has increased remarkably (x2.5) at the same time with the degree of increasing calcium deficiency (through the lack of osteoid mineralization).

11. Bone mineralization speed is strongly diminished in the calcium-deficient animals, as much as the deficiency degree increases, compared to the control group.

Bibliography


Înțelegerea modernă a etiologiei osteoporozei post-menopauză se bazează pe un dezechilibru între procesele de resorbție și formare a osului, dezechilibru dat de deficiența în hormoni estrogeni, care determină forme diferite și combinate de descreștere/creștere a activității celulelor osoase de tipul osteoblastelor și osteoclastelor. În acest sens se realizează analize histomorfometrice prin care se determină cantitatea relativă de os mineralizat și de osteoid, ca și activitatea celulelor cu funcție de formare (osteoblastele) sau de resorbție a țesutului osos (osteoclastele). Societatea Americană pentru Cercetarea osului și mineralelor recomandă evaluarea următorilor parametri histomorfometrici: volumul trabeculelor osoase (BV/TV, % - procentajul țesutului osos într-un volum dat. Schematic, el reprezintă raportul plin/gol în os); suprafața osteoidului (OS/BS, % - procentajul suprafeței de os format într-o suprafață de os dat); suprafața osteoblastelor (Ob. S/BS, % - procentajul suprafețelor trabeculare sau travelor osului spongios prezintând osteoblaste morfologic active); volumul osteoidului (OV/BV, % - procentajul osteoidului într-un volum de os dat); grosimea osteoidului (O. Th, m, - grosimea medie a benzii de osteoid, care reprezintă suportul calcificării); suprafața osteoclastelor (Oc. S/BS, % - procentajul suprafețelor trabeculare prezintând lacune de resorbție ocupate de una sau mai multe osteoclaste, cunoscut fiind faptul că, carențele în calciu determină creșterea numarului osteoclastelor și stimulează activitatea lor); rata mineralizării prin apozitie (MAR, m/zi), calculată prin divizarea calculată prin divizarea lungii mineralizare. Acest dublu marcaj a fost realizat prin injectarea calcinei i.m. la interval de 6 zile, 48 de ore înainte recoltării probelor. Cercetările relatate în prezent lucrare fac parte din subcontractul CEEX nr.110-2 partener nr.2 din cadrul contractului CEEX 110 intitulat „Model experimental pentru studiul biodisponibilității unor factori nutritionali (Ca, B, fitosteroli) și influența lor asupra mineralizării osului la porc, suport științific în studiul osteoporozei”.  

Cuvinte cheie: osteoporoza, mineralizare, osteoblaste, osteoclaste, osteoid, histomorfometrie.