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Immunohistochemical analysis of bone metabolism in osteonecrosis of the femoral head

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Abstract

Osteonecrosis of the femoral head occurs because of the suppression of blood circulation. At the level of the area affected by the ischemic phenomenon, there are two types of events, in the first stage there is necrosis of the cellular elements, followed by a reparatory phase of the spongy bone tissue. The objective of the study was the histological and immunohistochemical (IHC) analysis of bone metabolism in the repair phase. We observed the action of the main cells involved in the remodeling, the osteoblasts and the osteoclasts, following the reaction of the markers of their activity: osteoprotegerin, osteonectin, osteopontin. We included 23 patients diagnosed with femoral head osteonecrosis, stage II, Ficat and Arlet classification, biological material required for histological and IHC analysis being obtained during hip arthroplasty. Regardless of the age or presence of risk factors, the reaction to osteoprotegerin was mildly positive, being only highlighted at the level of the reactive dividing line, being absent in the other areas, indicating a reduced activity of inhibiting differentiation and activation of osteoclasts, also highlighted with classical histology methods, the affected area being well-defined and we could observe the necrotic tissue resorption by osteoclasts. The intense positive reaction of osteopontin and osteonectin, especially at the line of demarcation, is due to the increase in the number of osteoblasts required for the synthesis of neoformation bone tissue. We believe that the aspects revealed by our study can be a track in finding new-targeted therapies useful in stopping the development of the disease.

Keywords: osteonecrosis, repair phase, osteoblasts, osteoclasts.

Introduction

Osteonecrosis of the femoral head appears because of suppression of blood circulation at this level. At this level affected by the ischemic phenomenon, two types of events follow, in a first stage there is necrosis of the cellular elements, which is followed by a restorative phase of the spongy bone [1–3]. Changes in bone metabolism occurring in the second phase of this process are the subject of this research.

Bone resorption is the first event occurring in the repair phase, followed by a neo-bone formation step [1, 4–8]. The healing process, repairing the tissue affected by ischemia, is initiated at the junction between the area of necrosis and the area containing viable tissue, where the blood circulation is intact, the hyperemic zone [1, 2, 4].

In order to limit and counteract the effect of aggression, a reactive line, an interface of the two areas, is interpolated to the interaction between the necrosis-affected area and the adjacent viable tissue area. At the level of this demarcation area, there are mesenchymal and capillary cells that proliferate and migrate alongside macrophages and fibroblasts at the level of necrotic area [1, 2, 4, 9].

The progressive loss of mechanical support due to resorption of spongy bone tissue at the reactive interface between necrotic and viable bone tissue leads to a compensatory increase in the production of adjacent spongy bone tissue by stimulation of osteoblastic activity and formation of new trabecular bone [5–8].

Often, however, bone tissue neoformation fails to keep pace with bone resorption due to osteoclast activation, resulting in significant loss of bone tissue at the level of the subchondral plateau. Subsequent aggression to this area leads to fractures in the subchondral bone plate and the destruction of overlying cartilage, thereby compromising the functionality of the affected hip joint [2, 4, 5, 7].

The objective of this study was histological and immunohistochemical (IHC) analysis of bone metabolism in the repair phase in patients diagnosed with osteonecrosis of the femoral head. We observed the activity of the main cells involved in the remodeling phase, the osteoblasts and the osteoclasts, monitoring the reaction of the markers of their activity – osteoprotegerin, osteonectin, osteopontin.

Patients, Materials and Methods

The present study obtained the approval from the Ethics Committee of the University of Medicine and Pharmacy of Craiova, Romania. Twenty-three patients diagnosed with femoral head osteonecrosis, stage II, Ficat and Arlet classification, who underwent partial or total hip arthroplasty, were included in the study in the Clinic of Orthopedics–Traumatology, Emergency County Hospital,

Craiova, during 2014–2015, thus obtaining the biological material necessary for histological and IHC analysis.

The reason for the study of patients diagnosed in stage II was the fact that histological changes occurring at this stage are characteristic of the repair phase. From the patient charts, we obtained information on age, gender, reasons for consult, patient history, personal physiological and pathological history, behavior, as well as data obtained through the objective examination, clinical and paraclinical investigations were retained.

The biological material, consisting of bone fragments containing both the necrosis-affected area and adjacent areas, was fixed in formalin, fractionated, decalcified with 10% ethylenediaminetetraacetic acid (EDTA), pH 7.4, for three weeks old, included in paraffin, then stained with the Goldner–Szekely trichrome (GS trichrome) and Hematoxylin–Eosin (HE) methods, to visualize the microscopic aspects of the damaged area and to assess the extent of the changes occurring in the adjacent areas.

Depending on images obtained by classical histological methods, samples were selected for IHC highlighting of tissue antigens. Of all the selected blocks included in paraffin, sections of 4 μm were cut using a microtome (Microm HM350) equipped with a Section Transfer System (STS).

The method used for the IHC highlighting of tissue antigens was a two-stage method, based on a polymeric network viewing system (DAKO EnVision). In order to begin the immunohistochemistry sequences, the sections were first dewaxed in three successive xylene baths, rehydrated by washing in decreasing concentration of alcohols.

The actual immunohistochemistry technique contained a standard algorithm, with some variations depending on the antibodies used (Table 1).

Table 1 – ***Antibodies used in the immunohistochemical study***

Antibody	Clonality	Clone	Dilution	Manufacturer
Osteoprotegerin	Polyclonal	Polyclonal	1:50	Santa Cruz
Osteonectin	Monoclonal	15G12	1:40	Novocastra Leica
Osteopontin	Monoclonal	OP3N	1:50	Novocastra Leica

Results

In our study, 23 patients, 17 men and six women, aged between 23 and 61 years old were included. Patients included in the study had various risk factors, such as trauma in their history, alcohol consumption, smoking, corticosteroid therapy, renal transplantation, hematological conditions, but there were also patients with no risk factor.

All patients enrolled in the study were diagnosed with stage II femoral osteonecrosis, Ficat and Arlet classification. They were observed, regardless of age, gender or risk factors presented by patients, the histological and IHC aspects specific to the repair phase of the disease.

On classical histology samples, we noticed in most patients the existence of a demarcation line at the interface between necrotic bone tissue and the adjacent tissue. In the immediate vicinity of this demarcation line, numerous osteoblastic bone cells are distinguished from the lack of this type of cell in the rest of the adjacent areas (Figure 1).

Other structures viewed at the level of the reactive zone are neo-formative blood vessels, in the lumen of which red blood cells are observed. At this level, the Haversian canals appear enlarged, some of them showing fibrotic tissue in the interior, but also vascularization around (Figure 2).

Perilesional tissue appears condensed, at this level, cellularity is diminished and broken bone trabeculae that penetrate into this area can be visualized. In the perilesional tissue, there are dilated Havers canals, the bone lacerations being structurally sound (Figure 3).

The decrease in the number of mature bone cells in osteocytes is another common occurrence in the studied patients. In the affected area and its proximity, we can notice the disappearance of bone tissue, its replacement by the fibrous tissue, as well as being able to reveal necrotic tissue islands. We can see that the osseous blades that apparently integral aspect are embedded in the area of fibrinoid necrosis. The bone blades have a hypertrophic appearance, with many neoformation bone cells in their immediate vicinity (Figure 4).

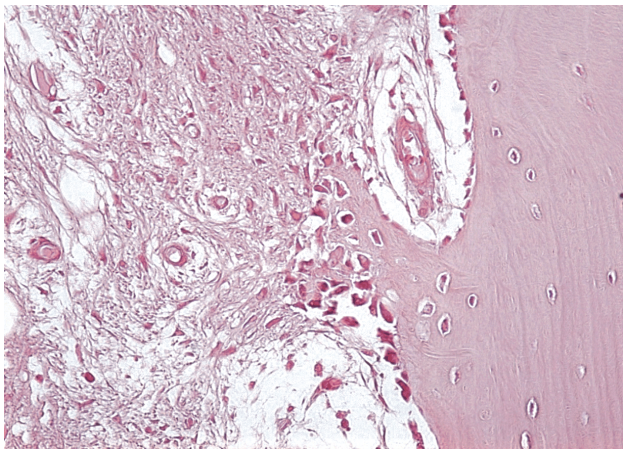

Figure 1 – ***The boundary between the healthy bone tissue and the area of necrosis, a reactive line where many osteoblasts can be seen (HE staining, ×200).***

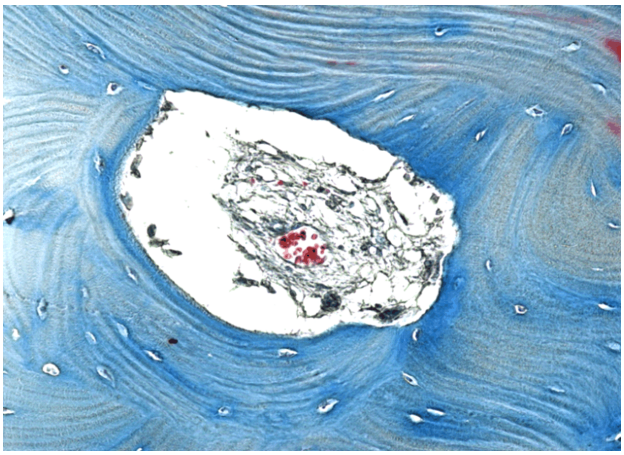

Figure 2 – ***Haversian canal with extensive lumen, with internal fibrosis and neoformation blood vessels (GS trichrome staining, ×200).***

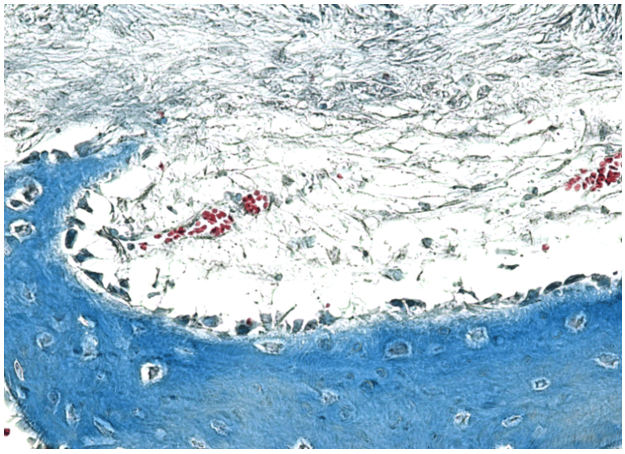

Figure 3 – ***Bone lamina, surrounded by hypertrophic osteoprogenic cells, penetrating the lesion area. It can also be seen neoformation blood vessels rich in red blood cells (GS trichrome staining, ×200).***

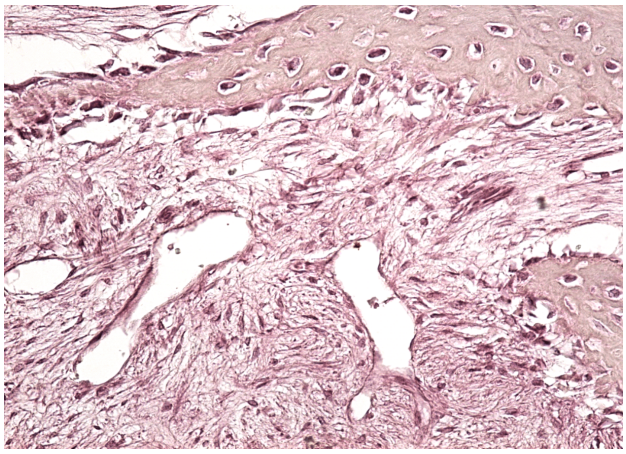

Figure 4 – ***Demarcation line between a healthy bone lamina, surrounded by many high activity osteoprogenic cells and the necrotic fibrinoid area (HE staining, ×200).***

The reaction to osteonectin was positive at the level of the overhaul area, with some patients experiencing a very intense reaction at this level. We also noticed an intense recall at the interface between bone and cartilage in patients where the lesion stretched to this level. In the lesion area with disorganized tissue, necrotic and fibrotic, as well as in the immediate vicinity of this area, the osteonectin reaction was negative in all patients included in the study (Figures 5 and 6).

The reaction to osteoprotegerin was poorly positive in all patients enrolled in the study, being only highlighted in the demarcation area and absent in other areas (Figures 7 and 8).

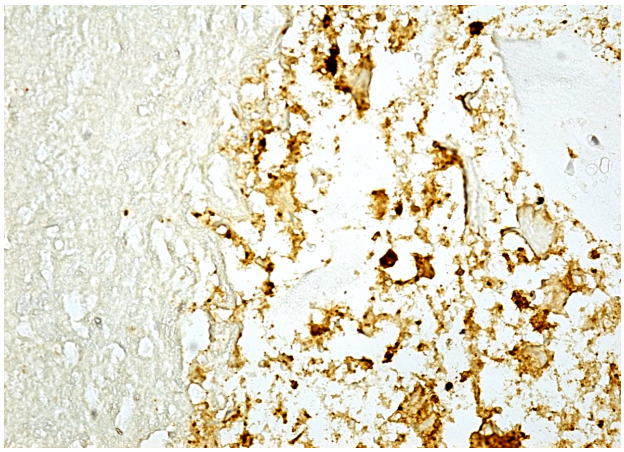

Figure 5 – ***Intensely positive reaction in the area of demarcation between damaged tissue and adjacent area where the repair process is initiated (Anti-osteonectin antibody immunostaining, ×200).***

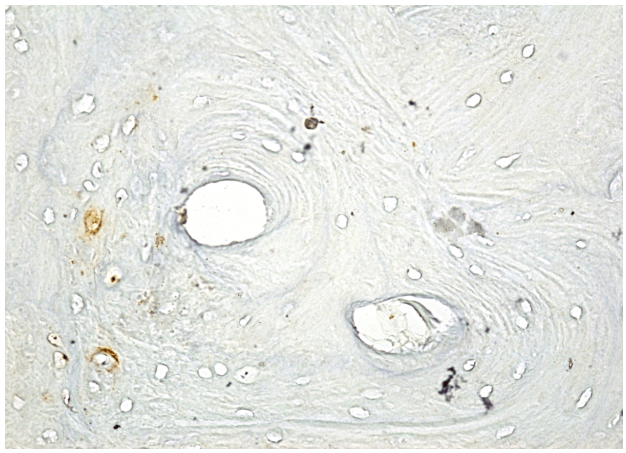

Figure 6 – ***Negative response, Haversian canals with widened lumen, cell-free bone lacuna (Anti-osteonectin antibody immunostaining, ×200).***

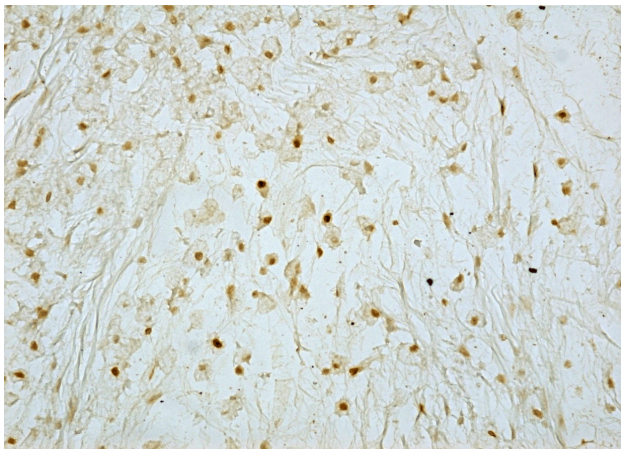

Figure 7 – ***Slightly positive reaction at the level of fibrinogen necrosis, disorganized tissue aspect (Anti-osteoprotegerin antibody immunostaining, ×100).***

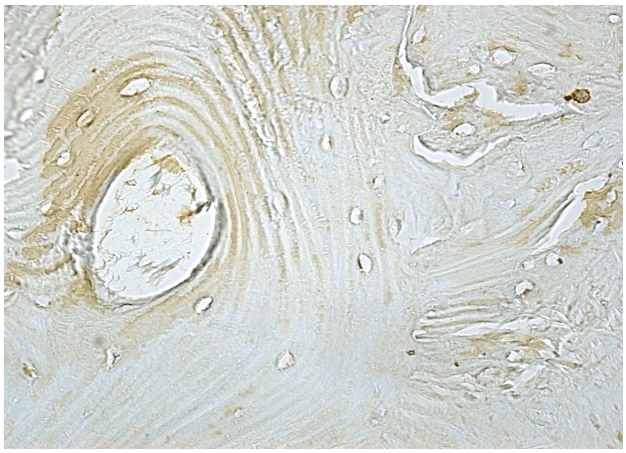

Figure 8 – ***Absence of reaction, necrotic tissue, empty Havers canal, absence of cellularity (Anti-osteoprotegerin antibody immunostaining, ×200).***

Immunoassay with osteopontin was absent or very weak near the necrotic area, instead in the reactive line was present. In some patients, an intense positive intracellular reaction was observed in the Havers channels and osteoma. At the level of the reshaping region, the reaction was very strong, especially at stellate-like cells, and was intense (Figures 9–11).

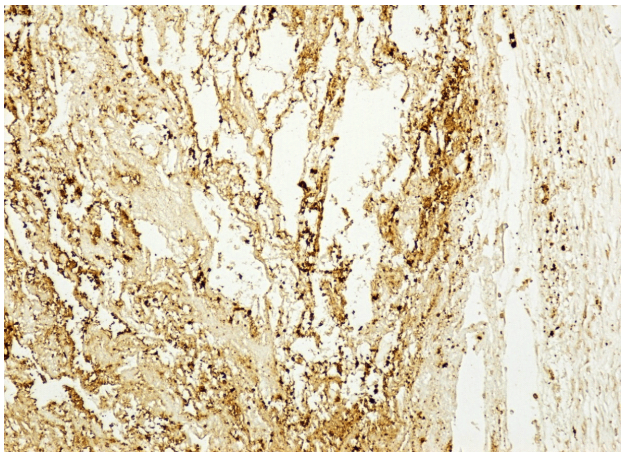

Figure 9 – ***Positive reaction, bone tissue undergoing reshape (Anti-osteopontin antibody immunostaining, ×100).***

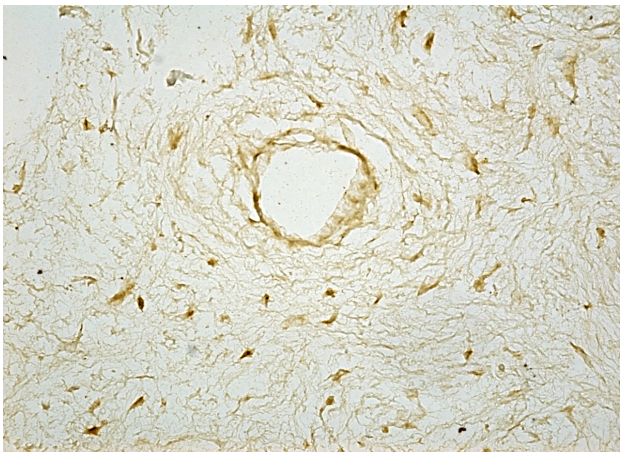

Figure 10 – ***Cells with positive immunomarking, stellar osteoblasts with intense activity (Anti-osteopontin antibody immunostaining, ×100).***

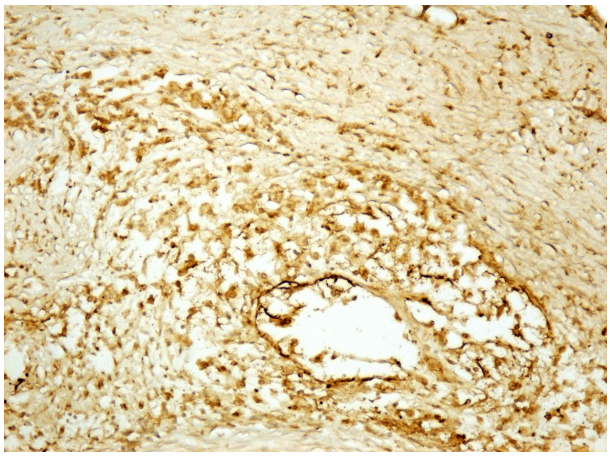

Figure 11 – ***Fibrosis area, weak positive reaction, more intense but in the lumen of the residual, intracellular Havers (Anti-osteopontin antibody immunostaining, ×100).***

Discussions

In patients diagnosed in evolutionary stage II, other histological changes similar to those described by us were noted in other studies. The appearance of bare bone gaps in the bones, the presence of poorly vascularized fibrous tissue areas entering the necrotic area as well as a considerable number of cement bands in the trabeculae showing areas of high bone remodeling are some of the things that are similar [10, 11].

Similar aspects found in the literature were the demarcation of the necrotic area with that of the reshuffled tissue, at the junction between the two areas, the proliferation of the capillaries, accompanied by fibroblasts, which then penetrated in the affected area. As a starting point, there is a decomposition of necrotic tissue by osteoclasts, as well as the emergence of newly formed bone osteoblasts.

All of these histological changes are encountered in the repair phase of the disorder [12].

Histopathological changes similar to those described by us have been reported in other papers, such as: decrease of cellularity near the affected area, replacement of necrotic tissue by newly formed bone fragments surrounded by granulation tissue, lack of bone osteocytes [13, 14].

The coupling of osteoresorption with osteoformation is the basis for the restoration of bone metabolism in the repair phase of femoral head aseptic osteonecrosis. For the IHC study, we selected three antibodies to show bone cell activity.

Osteoprotegerin is a member of the tumor necrosis factor superfamily, which plays a key role in bone remodeling.

Bone remodeling is controlled by a system of three proteins: receptor activator of nuclear factor kappa B (RANK), a transmembrane receptor-like osteoclast protein, and its ligands – osteoprotegerin and RANK ligand (RANKL), the two cytokines and osteoprotegerin RANKL, being competitors for the same receiver, RANK.

Osteoprotegerin is an osteoblast-secreted glycoprotein with a greater affinity than RANKL on the RANK receptor. Fixing osteoprotegerin at the RANK level translates into inhibiting bone resorption by blocking osteoclastogenesis and reducing mature osteoclast activity [15].

In the patients included in our study, irrespective of age or risk factors, the osteoprotegerin reaction was poorly positive, only visible at the demarcation area of the reactive line, absent in other areas, indicating a reduced activity of inhibition of the differentiation and activation of osteoclasts, as well as the classic histology methods, the affected area being well defined and the necrosis tissue resorption by osteoclasts.

Poor osteoprotegerin prominence in patients included in our study signifies increased bone resorption activity of affected bone tissue by osteoclasts as an event characteristic of the repair phase.

Osteoprotegerin has been identified as one of the main factors leading to bone balance/osteoclast balance, which has a role in bone remodeling [16].

In another study, osteoprotegerin deficient expression was detected in both the necrotic zone and the reshaping area, with no statistically significant differences. In the same patients, the expression RANK and RANKL, less than that of osteoprotegerin, were highlighted in both studied areas [17].

The role of osteoprotegerin in the repair phase in patients with osteonecrosis of the femoral head was also demonstrated in other scientific papers, with differences between patients presenting different risk factors. This demonstrates that the expression is lower, and therefore the bone resorption process becomes more pronounced in corticosteroids using variable duration [13].

Osteopontin (OPN), is a an important representative of the noncollagenous extracellular matrix and it is produced by osteoblasts and a cytokine involved in proliferation, apoptosis, and signaling inflammation, is implicated in bone reshuffling after physical aggression. The presence of OPN on the surface of the bone is very important for the reshaping of mature bone tissue [18]. Osteopontin (OPN), a phosphorylated sialoprotein, is an important member of the mineralized extracellular matrix of bones [19].

Osteonectin, secreted protein acidic and rich in cysteine (SPARC), is an extracellular matrix protein found in effective overhaul in the bone structure and other tissues. Osteoblasts secret SPARC in the neoformation of the bone tissue [20]. This mature protein is discriminatory bound to hydroxyapatite, collagen fibers, and vitronectin in specific locations and allows appropriate management of the bone matrix using connections with the surface of the cell.

Osteopontin and osteonectin were highlighted in the patients diagnosed with osteonecrosis of the femoral head included in the IHC study, indicating an increase in the number and intensity of osteoblast activity.

We made the immunoassays with osteopontin and osteonectin to highlight bone mineralization. The strong positive reaction, especially at the demarcation line, is due to the increase in the number of osteoblasts required for the synthesis of neoformative bone tissue.

In other papers, it has been shown that osteopontin and osteonectin are markers of bone remodeling encountered in the reparatory phase in patients diagnosed with osteonecrosis of the femoral head [20].

In another study, the strong osteocalcin staining was observed in osteoblasts in the patients diagnosed with avascular necrosis of the femoral head [10].

A similar study has shown an increased expression of factors regulating bone formation and remodeling in the femoral head and/or neck of patients with osteonecrosis of the femoral head [21].

Conclusions

All patients enrolled in the study, regardless of the risk factors presented, showed intense stimulation of osteoblasts, and minimal inhibition of osteoclasts. The increased bone remodeling capacity, as well as the reduction of bone density in the spongy bone tissue, characterize the repair phase. We believe that the aspects revealed by our study can be a starting point in finding new-targeted therapies useful in stopping the development of the disease.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Kamal Constantin Kamal and Daniela Teodora Maria equally contributed to this article.

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