Histopathological and immunohistochemical aspects of bone tissue in aseptic necrosis of the femoral head

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Abstract
Femoral head osteonecrosis, also known as avascular necrosis, is a disease with a multifactorial etiology, characterized by a profound change of bone architecture, which leads to the diminishing of bone resistance and femoral head collapse. The main causes that lead to femoral head necrosis are represented by the decrease of local blood perfusion and increase of intraosseous pressure, because of an excessive development of adipose tissue in the areolas of the trabecular bone tissue in the femoral head. The histopathological and immunohistochemical (IHC) study performed by us showed that most of bone trabeculae were damaged by necrotic-involutive processes, their sizes being reduced, both regarding their length and their diameter; generally, the spans were thin, fragmented, distanced among them, which led to the occurrence of some large areolar cavities, full of conjunctive tissue, rich in adipocytes. Some of the residual bone spans even presented microfractures. In the structure of the trabecular bone tissue, numerous cavities showed lack of content, which indicates the death of osteocytes inside, while the endosteum appeared very thin, with few osteoprogenitor, flattened, difficult to highlight cells. The IHC study showed a low reaction of the bone reparatory processes and a reduced multiplication capacity of bone cells involved in the remodeling and remake of the diseased bone tissue. Nevertheless, there were identified numerous young conjunctive cells (fibroblasts, myofibroblasts), positive to proliferating cell nuclear antigen (PCNA), cells that have a high capacity of multiplication, participating in the formation of a fibrous conjunctive tissue (sclerous) instead of the damaged bone trabeculae. The formation of fibrous conjunctive tissue causes the reduction of mechanical resistance of the femoral head and its collapse. The IHC study of the microvascularization in the femoral head damaged by aseptic osteonecrosis showed the presence of a very low vascular system, both in the residual bone trabeculae and in the sclerous conjunctive tissue. Of the inflammatory cells present in the spongy bone tissue of the femoral head affected by osteonecrosis, the most numerous ones were the macrophages. Both macrophages and T- and B-lymphocytes had a heterogenous distribution.

Keywords: avascular necrosis, femoral head, osteonecrosis, microcirculation, microfractures.

Introduction
Femoral head osteonecrosis or “bone tissue death” is a pathological state characterized by the damaging of the spongy bone architecture and the reduction of mechanical resistance [1, 2]. As a result of the anatomical and histopathological (HP) changes, of the change between the proportion between the bone surfaces of the hip joint, the disease starts to clinically manifest by joint pain, decrease in the coxofemoral joint movement, with a partial or total functional impotence. Due to the etiopathogenic mechanisms involved in the disease, osteonecrosis is also called avascular necrosis, ischemic necrosis, subchondral avascular necrosis or aseptic necrosis of the bone [3]. Most studies show that the main causes leading to femoral head necrosis are represented by the decrease of local blood perfusion and increase of intraosseous pressure, because of an excessive development of adipose tissue in the trabecular bone tissue areoles of the femoral head [4].

The onset of the bone necrosis and the decrease of bone mechanical resistance is followed by the collapse of the femoral head and, ultimetely, to an osteoarthritis of the hip [5].

Non-traumatic osteonecrosis of the femoral head is associated with one or more risk factors, of which we mention the following: alcohol intake, corticoids treatment, presence of blood disorders (sickle-cell anemia, thalassemia, polycythemia, myeloproliferative diseases, hemophilia), pregnancy, chronic kidney failure, hypercholesterolemia, Cushing disease, etc. [6–10]. Still, in approximately 30% of the patients, the etiology is unclear and, as a result,
these cases are also called idiopathic osteonecrosis [11]. The fact that avascular necrosis of femoral head sometimes occurs in twins and familial groups suggests the fact that there may also be involved genetic factors in the disease etiology [12].

Despite the intense mineralization and its uncertain aspect, the bone is quite dynamic, being continuously subjected to a process of remodeling, through which there is provided bone adjustment to mechanical burdens, fracture healing, calcium hemostasis, etc. [13, 14]. Bone remodeling consists in the balance of two antagonistic processes: bone resorption, performed by osteoclasts and formation of new tissue, performed by osteoblasts. The balance between the formation and resorption of the bone depends on the action of various local factors (biomechanical stimulation, local vascularization) and systemic factors (hormones, cytokines, chemokines) [15–18]. In osteonecrosis, the etiopathogenic factors strongly alter the processes of bone formation and create conditions for damaging the trabecular bone. Numerous clinical studies and experiments tried to clarify the intimate mechanisms leading to bone necrosis, still the pathogenesis of femoral bone is not yet completely understood up to the present moment.

**Aim**

In the present study, we proposed to highlight the HP and immunohistochemical (IHC) changes of the bone tissue in the femoral head damaged by aseptic osteonecrosis.

**Materials and Methods**

The studied biological material was represented by fragments of bone tissue sampled from the femoral head obtained from 45 patients clinically and paraclinically diagnosed with femoral head aseptic necrosis. During the surgery for hip arthroplasty, there were sampled femoral heads that were introduced in a 10% neutral buffered formalin fixing solution for two weeks. Then, every femoral head was cut into thin longitudinal sections, about 2–3 mm thick, with a mechanical small dimension saw, equipped with a fine blade. The bone tissue sections were passed through a 5% trichloroacetic acid (TCA) decalcifying solution for six weeks, for reducing the mineralization of the bone tissue, in order to obtain homogeneous thickness that prevented any microscopic artifacts.

The biological material was finally stained with Hematoxylin–Eosin (HE) and with the green-light Goldner–Szekely (GS) trichrome. For the IHC study, the bone tissue sections were placed on poly-L-lysine (Sigma), in order to increase the adherence of the biological material to the histological slides.

In our study, we used the following antibodies: anti-proliferating cell nuclear antigen (PCNA) (monoclonal mouse anti-PCNA, PC10 clone, 1/100 dilution, Dako) for the study of proliferative cells; anti-alpha-smooth muscle actin (α-SMA) (monoclonal mouse anti-human SMA, 1A4 clone, 1/100 dilution, Dako) for the study of myofibroblasts; anti-cluster of differentiation (CD) 34 (monoclonal mouse anti-human CD34 Class II, QBE clone 10, 1/50 dilution, Dako) for the study of blood vessels; anti-CD3 (monoclonal mouse anti-human CD3, F7.2.38 clone, 1/25 dilution, Dako), for highlighting T-lymphocytes; anti-CD20 (monoclonal mouse anti-human CD20cy, L26 clone, 1/50 dilution, Dako), for highlighting B-lymphocytes; anti-CD68 (monoclonal mouse anti-human CD68, KP1 clone, 1/100 dilution, Dako), for highlighting macrophages. The analysis of the IHC reactions was a qualitative one, evaluating the staining intensity, thus: intense positive reaction (+++), when the reaction occurred in over 80% of the cells; moderate positive reaction (++), when the reaction marked 30–80% of the cells; positive reaction (+), when the reaction occurred in over 5–30% of the cells; very poor positive reaction (+/-), when immunostaining was present in less than 5% of the cells; absent reaction (-), when it could not be identified any cell with a positive immunomarking.

**Results**

In our study, we observed that the microscopic aspects of the trabecular bone tissue in the femoral head affected by aseptic osteonecrosis were extremely variable from one patient to another, even from one area to another in the same bone. Also, there was highlighted that the bone changes were present not only in the femoral head, but also in the neck, microscopic aspects that prove the fact that osteonecrosis is a disease that may affect larger areas from the femoral proximal epiphysis.
The areas of bone necrosis had different sizes and affected both the trabecular bone and the bone marrow, thus generating acellular areas, filled with necrotic material and residual bone trabeculae with an incomplete necrosis (Figure 1). The bone trabeculae around the necrosis areas presented an atrophic, disordered or even absent endosteum.

The overall study of the spongy bone in the femoral head showed that bone trabeculae were reduced in number, damaged by necrotic-involutive processes, fragmented, distanced between them, which led to the occurrence of some large areolar cavities, full of conjunctive tissue, rich in adipocytes (Figure 2). Some of the bone trabeculae presented microfractures, thus proving that these were not capable of handling the mechanic forces of the hip joint and were not able to restore their trabecular structure (Figure 3).

The detailed study of the trabecular bone structure in the femoral head with higher microscopic objectives highlighted that numerous osteoblasts were content-free, thus indicating the death of osteocytes inside (Figure 4), most probably because of the chronic ischemia to which the femoral head trabecular bone was subjected. Most often, residual osteocytes presented an eccentric, condensed, hyperchromatic and heterogeneously unedged nucleus; most of the time, the osteocyte cytoplasm was clear, uncolored, showing a significant change of the quantity and quality of the cytoplasmic organelles.

Also, there was observed that most trabeculae had a heterogenous structure, with more or less colored areas, which expresses a reduction of the bone mineralization, as well as a profound change of the bone structure, with the decrease of the protein and glycoproteic component that, physiologically, maintains the hydroxyapatite crystals in the bone structure (Figure 5). In some areas of the trabeculae, there was noticed a strong demineralization, therefore there could be highlighted the structure of the collagen fibers in their composition (Figure 6).

In all the studied cases, there was highlighted the lack of the canaliculi, an aspect that shows a disruption of the intercellular communication, but also of the osteocyte feeding possibilities, because the canaliculi constitute a true intraosseous circulatory system through which all the trabeculae osteocytes are supplied with oxygen and nourishing substances, regardless of its thickness.

Another microscopic aspect under study was the endosteum, a membrane, thin, conjunctive structure that lines up the areolar cavities of the trabecular bone. The endosteum appeared very thin, with few osteoprogenitor cells, arranged on a thin layer of collagen fibers; in some bone fragments, the endosteum was absent, while in other bone fragments, we found a discontinuous endosteum.

Physiologically, in any bone lesion there occur processes of regeneration and remodeling, which depend on local vascularization, on the formation and activation of new osteoblasts and osteocytes, cells that are capable of synthesizing ossein. In our study, the processes of bone repair and remodeling were quite limited, most probably because of the reduction of blood flow and the onset of chronic ischemia. Bone regeneration occurred in some areas of the endosteum, areas characterized by the presence of osteoprogenitor cells with a hypertrophic, round, ovalary or polyhedral aspect, with an enlarged nucleus and abundant, basophil cytoplasm (Figure 7). In the extracellular environment, these cells are capable of synthesizing and exert all the components from the biochemical composition of the bone matrix (collagenous, non-collagenous proteins and glycosaminoglycans).

Through the immunohistochemistry studies, we investigated the capacity of bone cell regeneration, the intensity and cellular composition of the inflammatory infiltrate, as well as the vascular component of the tissue damaged by osteonecrosis in the femoral head.

The study of the cellular multiplication was performed using the anti-PCNA antibody. PCNA is a well-preserved protein, found in all species of eukaryotes, in the nucleus of cells with a multiplication capacity, regardless of the stages of cellular cycle. As our images show (Figure 8), the reaction to PCNA was a negative one (-), both in the osteocytes from the bone trabeculae and in the endosteum; consequently, we considered that the negative reaction to PCNA may be caused by the death of bone cells. Nevertheless, in the areas of bone regeneration, the reaction to PCNA was intensely positive (+++), both in the endosteum and in the adjacent osteocytes (Figures 9 and 10). In some areas of bone necrosis, there was highlighted a positive reaction (+++) to anti-PCNA antibody, a reaction that shows a capacity of multiplication of these cells in a hypoxic environment (Figure 11).

The microscopic aspects of sclerous conjunctive tissue in some areas of femoral head remodeling led to our investigation of the involvement of myofibroblasts in the process of conjunctive reconstruction. For this, we used a specific marker for myofibroblasts, namely α-SMA, an essential characteristic of these cells. Myofibroblasts have a high multiplication capacity, but, just like the fibroblasts, they have a high capacity of extracellular matrix synthesis and secretion, which turns them into key players in the process of wound repair and healing. In our study, we found a low number of myofibroblasts in the fibrous conjunctive tissue that was formed in the process of bone necrosis restoration (Figure 12).

The analysis of the vascular network in the bone tissue of the femoral head affected by aseptic osteonecrosis was performed using an IHC marker specific to the endothelial cells, namely CD34. As it may be observed from our images (Figures 13 and 14), in the femoral head osteonecrosis there is a low vascular network, both in the residual bone trabecular and in the sclerous conjunctive tissue that was formed because of the processes of bone tissue restoration in the femoral head.

The study of the inflammatory reaction in the femoral head affected by aseptic necrosis showed that this reaction had a different intensity from one area to another. Of the inflammatory infiltrate cells, the most numerous were the macrophages. Still, the density of macrophages varied a lot from one HP piece to another, and even from one area to another in the same femoral head, as the intensity of the inflammatory infiltrate was heterogeneous. There were identified areas with an intense reaction (+++) of the macrophages, and also areas with a moderate reaction (+) (Figures 15 and 16). Regarding the reaction of T- and B-lymphocytes, this was similar to that of macrophages, namely there were identified areas with numerous T- or B-lymphocytes, and also areas with a low number of lymphocytes (Figures 17–20).
Figure 1 – Area of bone acellular necrosis with a heterogenous aspect and residual bone spans inside (HE staining, ×100). HE: Hematoxylin–Eosin.

Figure 2 – Microscopic image of trabecular bone from the femoral head, where we may observe the reduction of the number and sizes of bone spans (HE staining, ×40).

Figure 3 – Image of isolated, deformed bone trabeculae, with intratrabecular microfractures (HE staining, ×40).

Figure 4 – Trabecular bone tissue with numerous empty bone cavities, because of the death of osteocytes inside (HE staining, ×200).

Figure 5 – Overall image of a bone trabeculae where we may observe the heterogenous aspect of the bone and the presence of a low number of osteocytes (GS trichrome staining, ×40). GS: Goldner–Szekely.

Figure 6 – Image of an area in a trabeculae where there may be observed an almost complete demineralization of the bone tissue and the expression of collagen fibers in the structure of bone blades (GS trichrome staining, ×400).
Figure 7 – Area of endosteum with large, hypertrophied, reactive osteoprogenitor cells, specific to areas of bone restoration (HE staining, ×400).

Figure 8 – Microscopic image of an isolated bone trabeculae, with a negative reaction of the component osteocytes to anti-PCNA antibody. In the endosteum, there are observed rare cells with a poor positive reaction to anti-PCNA antibody (Immunomarking with anti-PCNA antibody, ×200). PCNA: Proliferating cell nuclear antigen.

Figure 9 – Fragment of bone tissue with numerous osteoprogenitor cells from the endosteum intensely reactive to anti-PCNA antibody, proving an intense mitotic capacity (Immunomarking with anti-PCNA antibody, ×200).

Figure 10 – Conjunctive cells with an intense reaction to anti-PCNA antibody, part of them being transformed into osteoblasts, thus proving their osteotransforming capacity (Immunomarking with anti-PCNA antibody, ×200).

Figure 11 – Image of fibrous tissue developed in the necrosis and restoration areas of the bone tissue, rich in fibroblasts, with an intense reaction (+++) to anti-PCNA antibody (Immunomarking with anti-PCNA antibody, ×200).

Figure 12 – Myofibroblasts present in a moderate number in the restored conjunctive tissue of the femoral head (Immunomarking with anti-α-SMA antibody, ×200). α-SMA: Alpha-smooth muscle actin.
Figure 13 – Image of sclerous tissue from the femoral head with rare blood vessels (Immunomarking with anti-CD34 antibody, ×100). CD34: Cluster of differentiation 34.

Figure 14 – Fibroadipose areolar tissue with low vascularization (Immunomarking with anti-CD34 antibody, ×100).

Figure 15 – Immunohistochemical image where we can observe the presence of many foamy cytoplasm cells, specific to macrophages, identified at the edge of the avascular necrosis area (Immunomarking with anti-CD68 antibody, ×200). CD68: Cluster of differentiation 68.

Figure 16 – Image of fibro-adipose tissue from the bone areoles with a low number of macrophages (Immunomarking with anti-CD68 antibody, ×200).

Figure 17 – Abundant inflammatory infiltrate, mostly formed of T-lymphocytes, present in a fibro-adipose area from the spongy tissue areoles affected by aseptic necrosis, arranged mainly perivascularly (Immunomarking with anti-CD3 antibody, ×200). CD3: Cluster of differentiation 3.

Figure 18 – Fibro-sclerous conjunctive tissue resulting from the restoration of the necrosed bone tissue, where we may observe the presence of a low number of T-lymphocytes (Immunomarking with anti-CD3 antibody, ×200).
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Figure 19 – Abundant perinecrotic inflammatory infiltrate with a nodular organization, mainly formed of B-lymphocytes (Immunomarking with anti-CD20 antibody, ×200). CD20: Cluster of differentiation 20.

Figure 20 – Area of areolar, perinecrotic conjunctive tissue, diffusely infiltrated with B-lymphocytes (Immunomarking with anti-CD20 antibody, ×200).

Discussions

Aseptic necrosis of the femoral head is a condition of invalidity, with an ever-rising prevalence all over the world, caused by a critical, progressive decrease of the blood flow in the femoral head. According to some recent studies, in the US every year there are between 10 000 and 20 000 new cases of femoral head necrosis. The total number of the patients recorded with this condition in the US is about 2–3 million [20, 21], while in China there are recorded about 8.12 million patients [22].

The medical expenses are extremely high for this condition. Only in the US there are spent about 1.6 million dollars for the surgical treatment of femoral head osteonecrosis and for the physical recovery of patients undergoing a hip arthroplasty.

Most frequently, the disease occurs in young adults aged between 30–50 years old and, in almost half of the cases, it affects both coxofemoral joints. All these clinical and epidemiological aspects led to the increase of studies on this condition, to the identification of the risk factors, their monitorization and a rapid treatment for preventing the occurrence of some pathological changes in the trabecular bone tissue of the femoral head, which may become irreversible [23]. As a result, the early diagnosis of femoral head osteonecrosis is essential for selecting the treatment methods and establishing the prognosis of the disease [24].

In our study, we aimed at highlighting the microscopic changes both in the bone structures, namely of the spongy bone trabeculae from the femoral head, as well as the changes of the conjunctive tissue from the spongy bone areoles, as the two morphological structures are both structurally and functionally interconnected.

The areas of bone necrosis (bone infarction) equally affected both the trabeculae and the bone marrow.

Like other authors, we observed that the osteonecrosis lesions in advanced stages affect not only the femoral head, but also its neck, which makes us consider the fact that the disease affects more extended areas than the ones having a radiological expression [25–27]. In our study, we observed a major damage of the bone trabeculae morphology, which shows a change of the bone cell metabolism that find themselves in the impossibility to regenerate any qualitative bone tissue. We also consider that femoral head osteonecrosis is the result of local ischemia that causes important changes in the trabecular bone tissue [28]. Thus, according to some studies, when the ischemic limit is reached, osteocytes undergo atrophy and die, which leads to a higher number of empty osteoblasts. Releasing the lysosomes and activating the hydrolase enzymes acidifies the surrounding tissue and releases free fatty acids, which saponify with extracellular calcium in order to form soluble soaps [29].

The pathological mechanisms that lead to the onset of ischemia are extremely varied: local thrombi, fatty emboli, nitrogen bubbles, abnormalities of red cells, and other [9, 30]. Moreover, blood vessels may be affected directly through processes of vasculitis caused by irradiation or toxic factors [31]. Also, it seems that osteonecrosis occurs and aggravates with age [32].

In our study, we brought microscopic evidence that show the fact that the bone microarchitecture of the femoral head was quite altered, especially in the advanced stages of the disease, the necrotic and sclerotic areas of the femoral head being the areas with the poorest biochemical properties, favoring the collapse of the femoral head.

Other studies also consider that the structural damaging of the trabecular bone has a strong impact on triggering the femoral head collapse [33, 34]. Some changes of the bone microarchitecture were reproduced in some experiments on animal models [35, 36]. These studies tried to catch the early HP changes of the disease, as the femoral head osteonecrosis should be diagnosed in the first stages, when the chances of stopping the progression and even the healing probability are higher. Some experimental studies on lab rats involving induced local ischemia showed that the first microscopic signs that indicate bone ischemia are observed in the medullary spaces, where there occurs a reduction of the cell nuclei coloring from the hematogenous marrow, starting from the second day after inducing ischemia. Subsequently, there occur large, round or ovalary spaces filled with adipocytes [3]. Fatty
marrow becomes more highlighted in comparison to the hematopoietic marrow, while the smaller vessels present signs of necrosis; subsequently, after about 15 days of ischemia, there appear the osteocyte voids, the osteoclasts being empty and the trabecular surface that normally is covered by endosteum becomes void of osteoprogenitor cells. There may be said that the bone structural change occurs quite late, a reason for which, when the clinical signs occur, bone lesions are already in an advanced stage.

In our study, we observed microfractures of the bone spans in all the patients, thus reducing the resistance and elasticity of the trabecular bone tissue even more. Most research showed that the fracture of bone spans in the femoral head are “tiredness fractures” caused by very low processes of bone regeneration [37–39]. In our images, we also highlighted multiple areas of incomplete fractures in the bone trabeculae, with no signs of bone regeneration. Therefore, we believe that the collapse of the femoral head is strongly associated with the changes of the osteoblast and osteocyte activity.

IHC study we performed included the highlighting some particular microscopic aspects of the bone tissue in the femoral head affected by aseptic necrosis. A first aspect investigated in the present study was the proliferation capacity of bone cells involved in the processes of bone regeneration. For the study of the osteoformation processes, we used an IHC marker involved in the cellular multiplication processes, namely PCNA. In our study, PCNA expression was quite low in the proliferative cells. Therefore, we believe that the collapse of the femoral head is strongly associated with the changes of the osteoblast and osteocyte activity.

The microscopic study performed revealed significant changes of the areoles content in the spongy bone tissue. In the necrosis area, there were identified bone, acellular more or less extended distracted, with a heterogenous structure and even fragments of islands of residual bone tissue. The bone trabeculae were damaged by necrotic-involutive processes, fragmented, distanced between them, which led to the occurrence of some large areolar cavities, filled with conjunctive tissue, rich in adipocytes. Some of the residual bone spans presented microfractures, thus proving the fact that these were not capable of handling the mechanical forces of the hip joint and did not have the capacity to restore their structure. In the structure of the bone trabeculae, numerous osteoblasts appeared free of content, which shows the death of osteocytes inside, most probably because of a chronic ischemia to which the spongy bone of the femoral head was subjected. The endosteum was quite thin, with few osteoprogenitor, flattened cells, difficult to highlight under a microscope. IHC study showed a poor reaction of the bone reparatory processes and a low proliferative capacity of the bone cells involved in the restoration of the bone tissue. Also, there was highlighted the presence of a very low vascular system, both in the residual bone trabeculae and in the sclerous conjunctive tissue. Of the inflammatory cells present in the femoral head affected by osteonecrosis, the most numerous were the macrophages, an aspect that indicates the presence of high quantities of tissular and cellular debris that required phagocytosis.

Conflict of interests

The authors declare that they have no conflict of interests.

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Conclusions

The microscopic study performed revealed significant changes of the areoles content in the spongy bone tissue. In the necrosis area, there were identified bone, acellular more or less extended distracted, with a heterogenous structure and even fragments of islands of residual bone tissue. The bone trabeculae were damaged by necrotic-involutive processes, fragmented, distanced between them, which led to the occurrence of some large areolar cavities, filled with conjunctive tissue, rich in adipocytes. Some of the residual bone spans presented microfractures, thus proving the fact that these were not capable of handling the mechanical forces of the hip joint and did not have the capacity to restore their structure. In the structure of the bone trabeculae, numerous osteoblasts appeared free of content, which shows the death of osteocytes inside, most probably because of a chronic ischemia to which the spongy bone of the femoral head was subjected. The endosteum was quite thin, with few osteoprogenitor, flattened cells, difficult to highlight under a microscope. IHC study showed a poor reaction of the bone reparatory processes and a low proliferative capacity of the bone cells involved in the restoration of the bone tissue. Also, there was highlighted the presence of a very low vascular system, both in the residual bone trabeculae and in the sclerous conjunctive tissue. Of the inflammatory cells present in the femoral head affected by osteonecrosis, the most numerous were the macrophages, an aspect that indicates the presence of high quantities of tissular and cellular debris that required phagocytosis.

Other cells involved in the local inflammatory process, including aseptic necrosis of the femoral head, are the lymphocytes. In our study, both T- and B-lymphocytes were quite reduced in number, which shows the presence of some local specific antigens.
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