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A RABBIT MODEL OF OSTEONECROSIS OF THE FEMORAL HEAD: FROM DISEASE INDUCTION TO AUTOLOGOUS STEM CELL TREATMENT

(PhD THESIS)

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ABSTRACT

Avascular necrosis of femoral head is a disease caused by the diminution of blood supply to the femoral head. It usually strikes between the ages of 30 and 60. However, it can affect anyone at any age. The etiologies can be divided roughly into two, which are traumatic, being most commonly displaced fractures of the neck of femur; and non traumatic, being systemic steroid use, hematological diseases, diabetes, kidney disease, alcoholism, gout and Gaucher's disease. Whatever the inciting factor is, the result is the same in all circumstances: necrosis of the bony trabeculae with empty lacunae and almost complete absence of hematopoietic marrow elements. Although avascular necrosis of bone may occur in any part of the skeleton without giving any symptoms; the femoral head has its own peculiarity. When osteonecrosis happens in a weight bearing area, as the bone dies, it becomes soft and cannot support the overlying cartilage. As the disease progress, cartilage collapse occur, joint surfaces become irregular and deformed resulting in a situation so-called arthritis necessitating arthroplasty. The aim of the treatment of osteonecrosis of femoral head is to diagnose the disease in early stages and prevent the development of arthritis. The evolving field of regenerative medicine offers promising treatment strategies using cells, biomaterial scaffolds, and bioactive factors, which might improve clinical outcome. In early stages of disease the disease, it is speculated that application of growth and differentiation factors, autologous bone marrow and stem cells would help to preserve the integrity of femoral head thus saving the patient from a troubled future.

The goals of this project were generating a rabbit model of osteonecrosis, defining the timing of the pathological changes and healing and analyzing the effects of treatment with mesenchymal stem cells. In order to develop osteonecrosis in the femoral head epiphysis, steroid injection was coupled with surgical ablation of femoral neck vessels and hip capsule. Histological examinations of the femoral heads to define the timing of damage were performed at the end of sixth, fourth and second weeks. According to the findings of these trials, a time schedule for the steps of the experiment was set. Bone marrow aspiration is performed to isolate of mesenchymal stem cells. One week after, the bilateral surgical ablation of femoral neck vessels and steroid injection was performed. At the third week, mesenchymal stem cells were inoculated into epiphyseal region of right femoral head and left hips were left as controls. Femoral heads were harvested at the fifth and seventh weeks.

In order to make a quantitative, the area of both necrotic and newly formed bone was calculated and the number of osteocytes and empty lacunae were counted. It is found that the transplanted specimens contained significantly more live osteocytes.

Our results showed that stem cells have a potential in the treatment of osteonecrosis of the femoral head. However, clinical studies with different rehabilitation protocols with long follow up periods should be performed to prove if stem cell therapy can speed the regeneration process in a femoral head affected by osteonecrosis before arthritic changes take place necessitating an arthroplasty.

CHAPTER 1

INTRODUCTION

DEFINITION OF OSTEONECROSIS

"The power of reproduction which Nature possesses, displays itself in a great variety of morbid cases, but in none of them more remarkably than in a certain disease of bone termed necrosis", wrote James Russell, the first Professor of Clinical Surgery at the University of Edinburgh in 1794. This was the first description of bone necrosis which was clearly septic in origin. It was not until a century later that Axhausen (1928) realized that bone necrosis could occur in the absence of infection. The 20th century has seen an increase in the incidence of aseptic necrosis of bone in deep sea divers, tunnel workers and in patients given corticosteroids for a variety of conditions including organ transplantation.

Osteonecrosis (ON) is a disease resulting from the temporary or permanent loss of blood supply to the bones. Without blood, the bone tissue dies, and ultimately the bone may collapse. If the process involves the bones near a joint, it often leads to collapse of the joint surface. Osteonecrosis is also known as avascular necrosis, aseptic necrosis, and ischemic necrosis.

Although it can happen in any bone, ON most commonly affects the ends (epiphysis) of the femur, the bone extending from the knee joint to the hip joint. Other common sites include the upper arm bone, knees, shoulders, and ankles. The disease may affect just one bone, more than one bone at the same time, or more than one bone at different times.

The amount of disability that results from ON depends on what part of the bone is affected, how large an area is involved, and how effectively the bone rebuilds itself. Normally, bone continuously breaks down and rebuilds, old bone is replaced with new bone. This process, which takes place after an injury as well as during normal growth, keeps the skeleton strong and helps it to maintain a balance of minerals. In the course of ON, however, the healing process is usually ineffective and the bone tissues break down faster than the body can repair them. If left untreated, the disease progresses, the bone collapses, and the joint surface breaks down, leading to pain and arthritis. It is now possible to diagnose osteonecrosis of bone before destructive changes become obvious radiographically, yet osteonecrosis remains a problem of management. It may affect patients who would generally be considered too young for total joint replacement, and so efforts have been made to treat osteonecrosis of bone more aggressively from an early stage in the hope of preserving joint congruity; results, however, remain unpredictable. The early diagnosis of osteonecrosis of bone is dependent upon an awareness of the conditions in which it can occur.

Histological Features and Pathogenesis of Avascular Osteonecrosis

The earliest microscopic signs indicative of bone ischemia are seen in the marrow spaces, where starting from the second day, there is loss of nuclear staining of marrow cells and large round and ovoid spaces filled with fat appear [60]. The fatty and hematopoietic marrow becomes then ghosted and the small vessels show evidence of necrosis (figure 1); after 15 days the osteocytic lacunae are empty and the trabecular surface is devoid of cells. At the border of the necrotic zone there is proliferation of capillaries accompanied by fibroblasts and foamy histiocytes, which are responsible for the breakdown of necrotic fatty marrow, while dead bone is partly removed by osteoclasts and substituted by newly formed trabeculae; alternatively woven bone is laid down on the surface of dead trabeculae. Phemister coined the term "creeping substitution" to indicate this slow replacement of aseptic dead bone in contrast with the massive resorption or sequestration of dead bone associated with osteomyelitis [144].

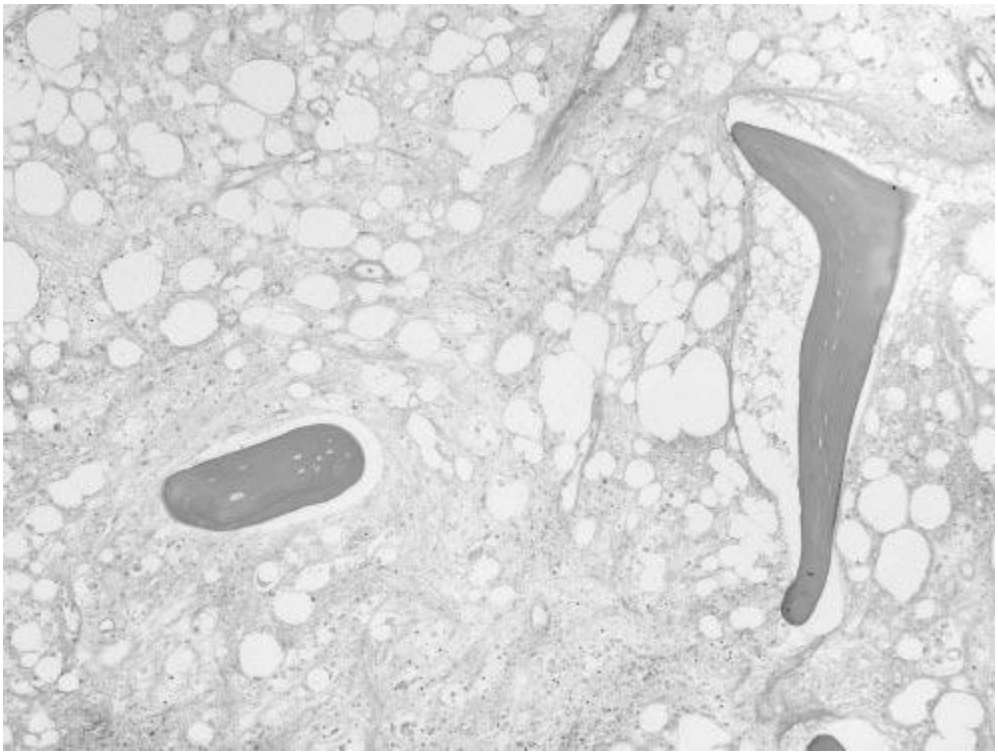

Figure 1 - ON following fracture of the femoral head. There is diffuse coagulative necrosis of marrow cells and osteocytic lacunae in bone trabeculae are empty (haematoxylin and eosin, 10x obj.).

The etiological factors of ON are known only in some conditions, like fracture, caisson disease, sickle cell disease, but less clear in others, like systemic lupus erythematosus,

corticosteroid administration, alcoholism; idiopathic forms are frequent as well. Osteonecrosis involves more frequently the convex articular surfaces because of the smaller diameter of the terminal vessels of this region and the absence of collateral vascularization. Other intrinsic factors contributing to the development of ON are the reduced vascularity of fat marrow in comparison with hematopoietic marrow and the non-extensive quality of bone tissue. Impairment of the blood flow may be caused by vascular compression (external pressure), trauma or occlusion of the vessels by nitrogen bubbles (in caisson disease) or rigid sickle cells (in sickle-cell anemia). The mechanism of ischemia and necrosis in other non-traumatic ON is not clear and several possible mechanisms have been proposed. Most authors think that bone necrosis results from primitive vascular problems, including vessel infarction, stenosing arteritis, arterio-sclerotic disease, extraosseous arterial involvement or extraosseous venous abnormality, or hypercoagulability and hypofibrinolysis. However, there is little histological evidence of vascular thrombosis, as there is little evidence of alterations in the coagulation mechanisms to support these theories. Frost has been the first to report the almost constant presence of trabecular microcracks in aseptic bone necrosis, with the possible exception of necrosis associated with work in compressed air, as the result of failure of healing of this microdamage with resultant fatigue fracture [57]. Diminished resistance of the affected bone is thought to determine secondary vascular impairment at the capillary level either through compression from relatively inelastic fat cells or through the rupture of small intratrabecular vessels. Interestingly, evaluation of transiliac bone-biopsy specimens from patients with aseptic ON and normal kidney function revealed a marked reduction in osteoblastic appositional rate and in bone-formation rate at the cell and tissue level, indicating that non-apparent metabolic disturbances are present in these patients [8]. Alterations in the remodeling of bone tissue may contribute to the development of ON in several ways, for example by inducing a healing defect of microfractures and thus facilitating subchondral fractures ultimately leading to ON [8].

Etiology

Osteonecrosis ular necrosis of bone is known to occur in a variety of conditions which include fractured neck of femur, traumatic dislocation of the hip, slipping of the upper femoral epiphysis and following osteotomy, disruption of the blood supply to the femoral head. However, in most of the non traumatic conditions, exact cause of osteonecrosis remains uncertain, whilst in others - such as rheumatoid arthritis and systemic lupus erythematosus - it

may be that the therapeutic regimen, such as corticosteroid administration, plays a part in bringing about osteonecrosis [70].

Fractures of the neck of the femur

Damage to the retinacular vessels supplying the femoral head and possibly tamponade due to bleeding into the confined joint space account for the 16% incidence of late segmental collapse of the femoral head in undisplaced united fractures and a 27% incidence in displaced fractures [13, 166, 194]. The incidence increases with the degree of displacement of the head; whilst there is probably no relationship with the number of manipulations or delay in treatment and open reduction, there is an increase in the incidence with extreme valgus reduction which may occlude the vessel of the ligamentum teres [62, 194]. Fractures of the femoral neck remain the commonest cause of osteonecrosis of the femoral head.

Traumatic dislocation

The overall incidence of osteonecrosis is 3.2% following anterior dislocation and 13.4% following posterior dislocation [54]. In 1962, Brav found that if the dislocated hip was reduced within two hours the incidence was 17.6%, whereas if greater delay occurred prior to reduction the incidence rose to 56.9%. Symptomatic osteonecrosis has been found to develop within two to three years [136].

Slipped capital femoral epiphysis

Slipping of the capital femoral epiphysis per se is less likely to produce osteonecrosis of the epiphysis than a forceful manipulation to reduce the slipped epiphysis [136]. The capital epiphysis slips posteriorly and therefore does not damage the posteriorly-situated retinacular vessels, whereas reduction of the slip may do so: hence the current move towards pinning of a slipped epiphysis in situ [69]. Again, the radiographic diagnosis of osteonecrosis may be made approximately six months following the slip. Acute cartilage necrosis is thought to occur in about one-third of patients with slipped upper femoral epiphyses [119]. The incidence of osteonecrosis lies between 6 and 16% [73]. This is thought to be increased by excessive traction on the leg, an early proximal osteotomy, and forceful manipulations.

Cervical osteotomy

The more proximal the osteotomy, the greater the incidence of osteonecrosis of the femoral head, ranging from 21% for cuneiform osteotomy of the neck to a reported incidence of 0%° following subtrochanteric osteotomies [143].

Sickle cell anemia

It may be that sickling, which occurs in response to lower oxygen tensions, occurs in areas of bone such as the epiphyses where the collateral circulation is limited. It is most commonly found in the femoral head but also occurs in the humeral head and rarely in the vertebral bodies causing collapse. It occurs in all types of sickle cell disease [14]. Sickle cell trait is not known to be a factor in ON unless associated with an abnormal form of haemoglobin [4].

Alcohol abuse

Alcoholics with liver disease do develop fatty emboli which may concentrate in the metaphyses of proximal and distal femur and tibia [85, 109]. The incidence of osteonecrosis in alcoholics is uncertain, but of 268 consecutive autopsy reports on chronic alcoholics with liver disease, 77.5% had demonstrable fat emboli in the lung, brain and other tissues [109]. The patient does not necessarily need to be an alcoholic or have liver cirrhosis. It is possible that increased fat deposition in the femoral head reduces the intraosseous blood flow, leading to bone ischemia and infarction. Alcoholics tend to deposit fat in a variety of areas including the femoral head and other bones [136].

Corticosteroid therapy

There have been two case reports of patients with Cushing's disease who developed osteonecrosis of the femoral heads, and in one patient the humeral head was also involved [110]. Patients given steroids for conditions such as pemphigus erythema multiforme, purpura, sarcoid, rheumatic fever and eczema, which themselves do not produce osteonecrosis; have developed osteonecrosis following steroid therapy [75]. Intra-articular injections of corticosteroids have been shown to predispose to Charcot's arthropathy which progressed to osteonecrosis.

Rheumatoid arthritis

Destruction of the hip joint is well known in rheumatoid arthritis and may be accelerated by the development of osteonecrosis. Edstrom found that 5% of patients with rheumatoid arthritis

developed osteonecrosis of the femoral head whether or not they had received steroids [48]. Most of the patients in his series who developed it had had rheumatoid arthritis for more than ten years. It may at times be difficult to distinguish between destruction of the joint by osteonecrosis and destruction by rheumatoid arthritis per se.

Systemic lupus erythematosus

Since steroids play a major role in the treatment of this disease, osteonecrosis has been attributed to the therapy rather than the disease [70]. However, isolated cases have been reported of patients with systemic lupus erythematosus who have never received steroids but who have developed osteonecrosis [47, 168]. Histological studies, with the exception of those of Velayos, have shown evidence of vasculitis in the synovial tissue but not the intraosseous vessels [185].

Chronic pancreatitis

Gerle et al. described 6 cases of osteonecrosis of the femoral head, the humeral head, femoral condyles and tibial plateau, but unfortunately described no histology [63]. All their patients were chronic alcoholics. In a series of autopsies of patients with acute and subacute pancreatitis, 10.4% showed bone marrow fat necrosis with no relationship between the presence of bone lesions and the level of serum amylase or the duration of pancreatitis [161].

Occlusive vascular disease

Isolated cases of osteonecrosis have been reported affecting various bones of the skeleton in cases of polyarthritis nodosa and advanced atherosclerosis and subacute bacterial endocarditis [29]. An interesting case was reported by Hirsch in 1938 of osteonecrosis of the femoral head, with histological evidence of thromboangiitis obliterans of the vessel of the ligamentum teres.

Osteomyelitis

Numerous cases are reported of osteonecrosis of the femoral head following osteomyelitis. Clearly, bone necrosis is a common occurrence in chronic bone infection.

Radiation

Vascular damage of the femoral head following radiation was first described by Ewing in 1926 who reported 3 cases. Histological study of fractures of the femoral neck following

radiation has failed to demonstrate osteonecrosis of bone, but does demonstrate marrow fibrosis and subintimal fibrosis and a reduced number of osteocytes in the bone [136].

Gaucher's disease

The cerebroside-containing cells infiltrate bone and frequently give rise to osteonecrosis, most commonly in the femoral head and often the femoral shafts [6]. This usually occurs bilaterally; between 40% of affected patients develop this complication [5, 6]. The natural history is of alternating remission and relapse.

Renal transplantation

Often multiple areas are involved by osteonecrosis of bone. Cruess et al. reported 9 cases of osteonecrosis of the femoral head in 27 patients who survived more than six months [40]. Nixon et al. in 1980, reported the incidence as 7.5% in 181 patients who had received 210 renal allografts [136]. Patients presented at an average of 30 months after transplantation, and those who developed this complication had on average four rejection episodes compared with one rejection episode in patients who failed to develop the condition. Patients who developed osteonecrosis of bone had on average received higher total doses of steroids during the first three and six months following transplantation. Following renal transplantation patients do tend to develop vague muscular aches and weakness, but the sudden onset of severe pain in one or more joints is very suggestive of the development of osteonecrosis of bone [135]. Children requiring transplantation tend to have severe pretransplantation renal osteodystrophy with growth retardation and sometimes epiphyseolysis as well as ON, whilst ON can also occur in patients on chronic hemodialysis [11, 176].

Caisson disease

The incidence of this condition rises with the number of dives. Under 300 dives, the incidence is 8.6% and over 900 dives it is 30.4% [120]. Gregg & Walder have reported its occurrence in 3.5% of North Sea divers [68]. The history of having had 'the bends' increases the risk of its development. Diving to more than 200 metres and suffering decompression sickness on the ascent appeared to lead to an increased incidence of bone lesions to as high as 16%, but radiology did not permit diagnosis until irreversible changes had occurred, nor did it give any indication of prognosis. There are two types: one is juxtaarticular and is potentially disabling; the second type occurs in the neck or shaft and is never symptomatic but is indicative of a failure of protective measures taken [136]. Bone isotope scans taken ten years after

discontinuing work in high-pressure environments can still demonstrate 'hot spots', sometimes in the absence of radiological progression of the lesion or in the presence of radiological normality, suggesting a reactive or repair process which may be prolonged and not necessary beneficial as structural failure of the joint may subsequently occur [67]. Compressed air workers have an 18% incidence of bone lesions of which 36% are juxtaarticular and 12% in the femoral head. United Kingdom commercial and naval divers have a 4% incidence of bone lesions of which 21% are juxtaarticular. Japanese fishermen who dive to catch fish and who are often males over the age of 50 have an incidence of 75% where decompression methods are poor [140]. The greater the number of exposures, the longer the exposures, the greater the depth and the occurrence of decompression sickness, all increase the incidence of dysbaric ON. There is no way to predict which lesions are going to remain static and which are going to progress [120].

Idiopathic

This was first described by Chandler (1948) as 'coronary artery disease of the hip' ('Chandler's disease'). Most of the patients described were alcoholics or had been treated with steroids in the past. The patients affected are usually middle-aged (range 45-60 years) with increasing pain in the hip. Males predominate by 4:1 and the disease is bilateral in 42-60% of cases [113, 142]. It most frequently affects the femoral heads but has been described as affecting the femoral condyles also. When the area of femoral condyle involved exceeds 2.3 cm2, the lesion tends to progress with the development of osteoarthritis [157]. When the hip is involved there is a sudden onset of pain in the groin, often with, initially, a normal radiograph. The other hip becomes involved in about 50% of patients within two years and hence provides excellent material for the study of the condition. Conditions associated with the development of idiopathic osteonecrosis of bone are gout and hyperuricemia, diabetes, hyperglycemia, minor congenital anomalies of the hip, obesity, the Leriche syndrome, coagulation deficiency, cretinoid epiphyseal dysgenesis, and dyschondroplasia. Charcot's disease and many other conditions have been described in association with osteonecrosis of bone.

Osteonecrosis of the Femoral Head

According to the experience of the Hospital for Special Surgery (New York), 20% of the femoral head resections for non-traumatic causes are performed for subchondral osteonecrosis; about 60% of idiopathic cases are bilateral and the majority of these patients have been treated with corticosteroids or are alcoholists [28]. The median age of patients treated for subarticular osteonecrosis is 54 years while that of patients treated for ostearthritis is 67. In a review of the experience of the Hospital for Joint Diseases (New York), from approximately 2000 total hip replacement carried out between 1984 and 1989, the presence of ON was identified in 345 patients (377 specimens, 18.9%) [169].

Before 1960, ON of the femoral head in absence of fracture of the femoral neck was considered an unusual event. In 1962, Mankin and Brower reviewed the literature and could find only 27 reported cases, while nowadays this condition is recognized as an important cause of osteoarthritis [113]. Most patients are males, 30 to 60 years old, and there is often history of corticosteroid treatment or alcoholism. The reported incidence of bilateral involvement is between 50% and 70% [113].

In addition, ON is observed as a secondary complication of osteoarthrosis of the hip in about 10% of femoral head removed for joint replacement [58, 197]. Secondary ON appears more frequently as a superficial “shallow” lesion involving 2-3 mm in depth beneath the articular surface (36.5% of cases), while less frequently the lesion is larger and shows a wedge shape similar to that of primary subarticular osteonecrosis [197]. The first type is probably a result of bone eburnation of osteoarthrosis, which interferes with blood flow, while the second type appears to be an independent event presumably caused by similar causal factors involved in primary ON [58, 197].

A fracture of the femoral neck is always followed by ischemic necrosis of the femoral head, due to the interruption of the blood supply. These fractures are occasionally seen in the young age following major trauma, but they are more common in the osteoporotic or osteomalacic bones of the elderly women after very minor trauma. Following the process of fracture repairing, or starting from the viable subfoveolar bone, the infarcted femoral head undergoes revascularization and reparative processes take place. This is sometimes followed by a collapse of the femoral head, a complication that usually occurs after a 1.5-2 year period from the fracture of the femoral neck, and that leads to late secondary changes [33, 82]. Catto has described the morphologic modifications associated with ON of the femoral head due to sub-

capital fracture, which according to her papers occur four different steps, but this description can applied to other forms of ON as well. In the first stage there is necrosis of the bone marrow, without evidence of reparative processes; in the second stage the reparative processes become evident at the periphery of the necrotic region; in the third stage there is collapse of the articular surface; and in the fourth stage there is evidence of secondary osteoarthritis [33, 34]. These classical anatomical descriptions form the basis of the classification schemes for staging of ON developed for imaging techniques:

– Stage I. At external examination the shape of the femoral head is unaltered, but on frontal section there is a wedge shaped subarticular area of necrosis in which the marrow is yellow-opaque. The infarct is well delimited from the surrounding bone by a thin hyperemic border. Histologically, the articular cartilage is viable, while the marrow elements of the subarticular bone are substituted by granular eosinophilic material and ghosts of necrotic adipocytes can be identified. The trabecular bone shows empty osteocytic lacunae and at the margin of the infarct there is increased osteoblastic and osteoclastic activity, accompanied by a proliferation of capillaries and fibroblasts in the marrow spaces, which correspond to the hyperemic rim seen macroscopically;

– Stage II. As in stage I, the articular surface is unaltered, but due to the progression of reparative processes, the infarcted area appears better demarcated on section (Figure 2), and a peripheral rim of sclerosis is visible radiologically. The proliferating capillaries and fibroblasts extend into the necrotic area, and dead bone trabeculae are removed by osteoclasts and substituted by viable new ones, laid down by osteoblasts, resulting in the sclerotic rim;

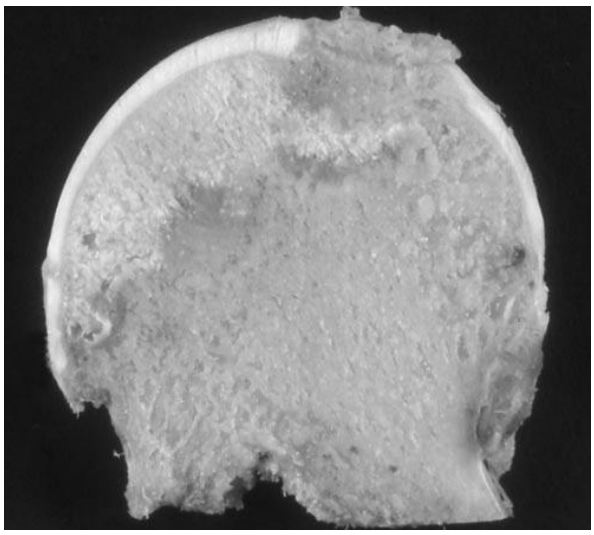

Figure 2 - Frontal section of a femoral head showing a wedge-shaped subarticular area of necrosis, which is well separated from the viable bone by a rim of granulation tissue. The convex shape of the femoral head is maintained.

– Stage III. At this stage there is an evident modification of the shape of the articular surface due to the collapse of the necrotic bone. On frontal section, there is a linear fracture usually located just below the articular cartilage, at the bony end plate level, or less frequently within the necrotic area or at its periphery (Figure 3). Histologically, the fracture area shows an admixture of fragmented bony trabeculae and cartilage with reparative tissues, including reactive woven bone, cartilage and granulation tissue, that is the appearance of unstable fracture elsewhere in the skeleton. Focal necrosis of the repair tissue around the area of fracture-collapse may occur and it should not be interpreted as recurrent ON, since it is likely not related to a recurrent ischemic event but rather to the injury resulting from the fracture itself [195];

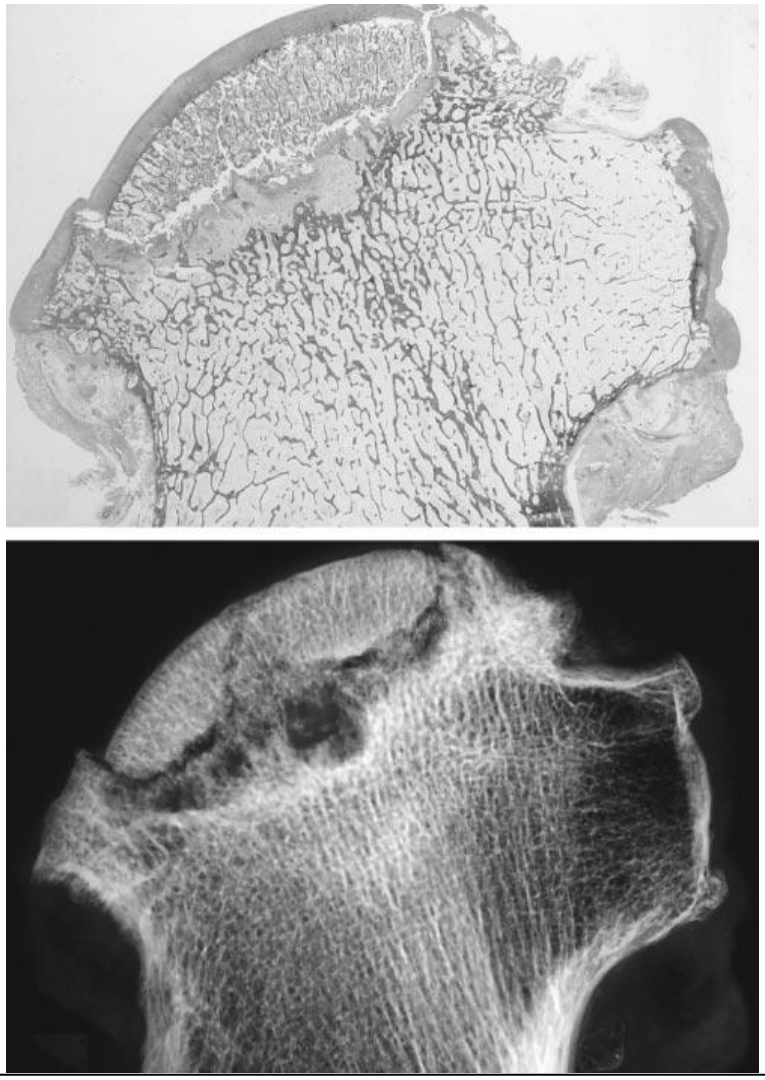

Figure 3 - A – Scanning view of a frontal section of an infarcted femoral head stained with haematoxylin and eosin. A subchondral fracture is present at the periphery of the infarcted area, which has caused the collapse of the articular surface. The trabeculae in viable bone adjacent to the infarct are thickened. **B –** Specimen radiograph of the same frontal section in A, showing the fracture line and the rim of bone sclerosis at the periphery of the infarct.

– Stage IV. At this stage the shape of the femoral head is severely deformed, due to the progressive detachment of bone and cartilage fragments from the infracted area (Figure 4). Bony and cartilaginous debris can be seen in the capsular and synovial tissues. This is accompanied by the development of signs of osteoarthritis, including eburnation of bone around the infarct. When osteoarthritic changes are fully developed, it may not be possible to recognize the initial cause as that of subarticular osteonecrosis.

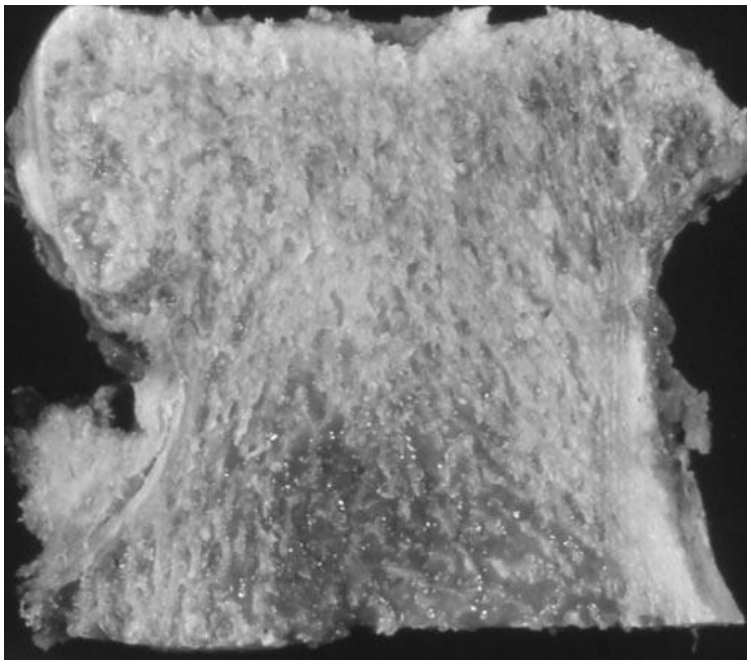

Figure 4 - Frontal section of a femoral head showing severe deformity due to the progressive detachment of bone and cartilage fragments from the infarcted area.

Treatment Modalities for the Osteonecrosis of the Femoral Head

Non-operative treatment

Non-operative treatments for ONFH are generally associated with less favorable results when compared with operative treatments [123]. Non-operative treatment, using bed rest and non-weight-bearing crutches resulted in 22.7% clinical success rate as compared with 53% for the core decompression group [123]. According to the literatures, the elimination or avoidance of risk factors was effective for the prevention of disease progression only in steroid-related ONFH and renal transplant patients [93, 179]. However, recently new modalities of non-operative treatment for ONFH have been developed with some promising results. Hyperbaric oxygen therapy was used in 12 patients with stage-I ONFH. Daily therapy of 100% oxygen at 2 to 2.4 atmospheres absolute in a hyperbaric chamber for a total of 100 sessions had resulted in 81% success rate by using MRI as the evaluation tool [151]. By following 284 patients

under high dose steroid therapy and concomitant use of statin for an average of 7.5 years, the lipid clearing agent seems to reduce the incidence of ON to 1% [147]. The anti-osteoporotic agent, bisphosphonate, has also been reported to be effective in modifying the natural courses of both traumatic and nontraumatic ONFH [95, 147, 150]. Theoretically, the disease could only be modified, or the time to collapse could only be delayed by bisphosphonates, because they only uncouple the bone remodeling process in the osteonecrotic femoral head. In contrast, a prostacyclin derivative iloprost was used in bone marrow edema or femoral ON and resulted in significant improvements both clinically and radiographically by MRI analysis [46]. Unfortunately, there is still no enough evidence to support the routine use of these pharmacologic agents for the treatment of ONFH in current practice. In addition, physical stimuli such as extracorporeal shock waves have also been reported as an effective treatment and inducing angiogenesis in the necrotic lesions [186, 187].

Operative treatment

Operative treatments for ONFH are miscellaneous and can be divided into joint-preservation and replacement procedures.

Joint replacement

Joint replacement procedures can be categorized as partial replacements or total replacements. Partial replacements replace the femoral heads and leave the innate acetabular cartilage to couple with a metallic ball head for joint functions. Unipolar replacements were associated with unsatisfactory results in more than 75% of patients because of acetabular cartilage wear. Hemi-resurfacing when performed on earlier stages of the disease had 61% good to excellent results in the intermediate term of follow-up [80]. To decrease the acetabular cartilage wear, bipolar prosthesis was designed to allow most of the joint motion between the inner polyethylene bearings [100]. However, clinical results of bipolar hemiarthroplasty for the treatment of ONFH remain controversial and leave the total hip replacement as the ultimate treatment choice [30, 35]. Unfortunately, the results of total hip replacements in patients suffering from ONFH are inferior to those with other diagnoses. Reasons responsible for the inferior results are not well elucidated but are usually attributed to the young and active life styles, the underlying risk factors, and abnormal bone quality affected by the disease. Recently, total resurfacing replacements have been reported for the treatment of ONFH in young active patients but the results are even more controversial [174]. It is likely that total

hip replacements can provide immediate pain-relief and good functional recovery of the patients. The negative impact on the implant durability may be minimized by the improvement of surgical techniques and choices of implants in the future.

Core decompression

Joint-preservation procedures are miscellaneous. Among them, core decompression is the most common procedure for early-stage ONFH [92, 123]. In an extensive review of 1206 hips treated by core decompression, Mont et al. reported an overall 64% satisfactory results as compared with 23% in the non-operative treatment groups [123]. Using the Ficat and Arlet classification, the result was good with 88% of stage I, 71% of stage II, and only 26% of stage III [127]. Using small diameter trephine for multiple drilling, Lee et al. achieved an overall 56% success rate (with 67% of the pre-collapse stage and 40% of the post-collapse stage) [101]. It was also shown that a large extent of the lesion (combined necrotic angle more than 200°), lateral location of the necrotic lesion, and higher intraosseous pressure in the intertrochanteric region were associated with unsatisfactory outcomes [101]. Core decompression can also be supplemented with pulsing electromagnetic fields or electric stimulation, but they are not routinely used in clinical practice because of inconclusive outcomes [116, 175].

Nonvascularized bone grafting

Nonvascularized bone grafting can be structural grafts or non-structural cancellous grafts. The advantages of this procedure include mechanical support to the femoral head, scaffolding for osteoconduction, and decompression of the necrotic lesion. Non-vascularized grafting techniques were often used as an adjunct to core decompression in the middle of the last century [20, 21]. The short-term results were good but they rapidly deteriorated after a mean of 14 years follow-up [125, 170]. To improve the results, many others reported new approaches such as the trapdoor technique, strut grafting and impaction bone grafting by the "light bulb procedure" [27, 91, 124, 153, 155]. In 13 of 15 hips (81%) treated by the "light bulb procedure", the hips were asymptomatic at a mean follow-up of 12 years [155]. Combination of local cancellous grafting and a wire coil implantation, with a mean follow-up of 61 months (range, 30 to 103 months), achieved a clinical success rate of 73% [203]. This technique can decrease the amount of cancellous grafts and provide mechanical support to the subchondral bone of the osteonecrotic femoral head.

Vascularized bone grafting

In addition to structural support and osteoconduction, vascularized grafting can provide viable cells for repair and a vascular channel to the ischemic femoral head. The reported three types of vascularized grafts are vascularized fibular, vascularized iliac, and muscle-pedicle grafts. Vascularized fibular grafting had an overall success rate of 50% to 80% when it was performed on both precollapse and early segmental collapse cases [86, 114]. Vascularized iliac grafting was reported to have 80% successful clinical results and 70% successful radiologic results in precollapse cases at 2 to 12.5 years follow-up [50]. Muscle pedicle grafting had 60% clinical success rate and 40% radiologic success rate in an average follow-up of 47 months [76]. For segmental collapse lesions, vascularized fibular grafting had 64.5% satisfactory results with a minimum follow-up of 5 years in a series of 224 hips [16]. In contrast, only 24% clinical success rate was achieved in 33 segmental collapse osteonecrotic hips with a mean follow-up of 74 months [36]. Possible reasons for the better results of vascularized fibular grafting may be that (1) the vascular pedicle is bigger and not kinked after rerouting to the femoral head, (2) the structural support of the cortical fibular strut to the subchondral bone is stronger, (3) the position of the fibular graft can be more ideally placed under fluoroscopic guide, and (4) the reparative potential of the fibular graft is more reliable than that of the iliac crest which could possibly be affected by extensive ON [36].

Alternative grafting

Alternatively, autologous bone marrow and bone morphogenetic protein could be used in combination with bone graft or demineralized bone matrix [77]. Wire coils, metal cages, porous tantalum rods, and polymethylmethacrylate cement have also been used to provide mechanical support and decrease the amount of grafts needed to fill the voids after debridement [167, 191]. Although the early clinical results are promising, no long term follow-up is available to justify their routine clinical use.

Osteotomy

Femoral osteotomy has been devised as a salvage treatment in younger and more active patients. The rationale of the procedure is to reduce the stresses on the necrotic zone and to prevent progressive collapse of the femoral head. Various types of osteotomies have been reported and two main types are intertrochanteric varus or valgus (combined with flexion or extension) osteotomies and transtrochanteric rotational osteotomies. The intertrochanteric

varus or valgus osteotomies are less technically demanding but the capacities for correction are relatively smaller as compared with the rotational osteotomies. For instance, when the lesion extends to the lateral third of the femoral head leaving an intact arc of less than 20, a varus osteotomy is inappropriate for moving the necrotic lesion away from the weight bearing area. In well-selected cases, treatments with varus osteotomy combined with flexion or extension, Mont et al. reported 76% good to excellent results at a mean of 11.5 years [126]. Similar results were achieved in 36 (80%) of 45 patients treated with valgus osteotomy and bone-grafting [162].

Transtrochanteric rotational osteotomy is more effective in repositioning the osteonecrotic lesion than varus or valgus osteotomies. The best results were reported by Sugioka et al. where a success rate of 78% was achieved in 229 of 295 hips followed for 3 to 16 years [178]. Similar results could not be reproduced by others and this procedure is considered to be highly technically demanding [42]. Reasons for the high incidences of failure can be attributed to technical difficulty, improper patient selection, inadequate fixation devices, undue rotation angles, too early weight bearing, and possible ethnic differences [42].

Regenerative Medicine

Regenerative medicine is a branch of translational research in tissue engineering and molecular biology which deals with the "process of replacing, engineering or regenerating human cells, tissues or organs to restore or establish normal function". This field holds the promise of engineering damaged tissues and organs via stimulating the body's own repair mechanisms to functionally heal previously irreparable tissues or organs.

Regenerative medicine also includes the possibility of growing tissues and organs in the laboratory and safely implants them when the body cannot heal itself. If a regenerated organ's cells would be derived from the patient's own tissue or cells, this would potentially solve the problem of the shortage of organs available for donation, and the problem of organ transplant rejection.

Attributed to having first been coined by William Haseltine (founder of Human Genome Sciences), the term "Regenerative Medicine" was first found in a 1992 article on hospital administration by Leland Kaiser. Kaiser’s paper closes with a series of short paragraphs on future technologies that will impact hospitals. One such paragraph had ‘‘Regenerative

Medicine'' as a bold print title and went on to state, ''A new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems.''

Regenerative medicine refers to a group of biomedical approaches to clinical therapies that may involve the use of stem cells [152]. Examples include the injection of stem cells or progenitor cells obtained through directed differentiation; the induction of regeneration by biologically active molecules administered alone or as a secretion by infused cells (immunomodulation therapy); and transplantation of in vitro grown organs and tissues (tissue engineering) [177].

Tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physico-chemical factors to improve or replace biological functions. While it was once categorized as a sub-field of biomaterials, having grown in scope and importance it can be considered as a field in its own right. While most definitions of tissue engineering cover a broad range of applications, in practice the term is closely associated with applications that repair or replace portions of or whole tissues (i.e., bone, cartilage, blood vessels, bladder, skin, muscle etc.). Often, the tissues involved require certain mechanical and structural properties for proper functioning. The term has also been applied to efforts to perform specific biochemical functions using cells within an artificially-created support system (e.g. an artificial pancreas, or a bio artificial liver). The term regenerative medicine is often used synonymously with tissue engineering, although those involved in regenerative medicine place more emphasis on the use of stem cells or progenitor cells to produce tissues.

Stem Cells for Therapy

Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can divide (through mitosis) to produce more stem cells. They are found in multicellular organisms. In mammals, there are two broad types of stem cells: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues. In a developing embryo, stem cells can differentiate into all the specialized cells; ectoderm, endoderm and mesoderm—but also maintain the normal turnover of regenerative organs, such as blood, skin, or intestinal tissues.

The classical definition of a stem cell requires that it possess two properties:

Self-renewal: The ability to go through numerous cycles of cell division while maintaining the undifferentiated state.

Potency: the capacity to differentiate into specialized cell types. In the strictest sense, this requires stem cells to be either totipotent or pluripotent—to be able to give rise to any mature cell type, although multipotent or unipotent progenitor cells are sometimes referred to as stem cells. Apart from this it is said that stem cell function is regulated in a feedback mechanism.

Two mechanisms exist to ensure that a stem cell population is maintained:

Obligatory asymmetric replication: a stem cell divides into one mother cell that is identical to the original stem cell, and another daughter cell that is differentiated.

Stochastic differentiation: when one stem cell develops into two differentiated daughter cells, another stem cell undergoes mitosis and produces two stem cells identical to the original.

There are three known accessible sources of autologous adult stem cells in humans:

Bone marrow, which requires extraction by harvesting, that is, drilling into bone (typically the femur or iliac crest).

Adipose tissue (lipid cells), which requires extraction by liposuction.

Blood, which requires extraction through apheresis, wherein blood is drawn from the donor (similar to a blood donation), and passed through a machine that extracts the stem cells and returns other portions of the blood to the donor.

Stem cells can also be taken from umbilical cord blood just after birth. Of all stem cell types, autologous harvesting involves the least risk. By definition, autologous cells are obtained from one's own body, just as one may bank his or her own blood for elective surgical procedures.

Adult stem cells are frequently used in medical therapies, for example in bone marrow transplantation. Stem cells can now be artificially grown and transformed (differentiated) into specialized cell types with characteristics consistent with cells of various tissues such as muscles or nerves. Embryonic cell lines and autologous embryonic stem cells generated

through Somatic-cell nuclear transfer or dedifferentiation have also been proposed as promising candidates for future therapies [182].

Stem cell therapy is the use of stem cells to treat or prevent a disease or condition. Bone marrow transplant is the most widely used stem cell therapy, but some therapies derived from umbilical cord blood are also in use. Research is underway to develop various sources for stem cells, and to apply stem cell treatments for neurodegenerative diseases and conditions, diabetes, heart disease, and other conditions. With the ability of scientists to isolate and culture embryonic stem cells, and with scientists' growing ability to create stem cells using somatic cell nuclear transfer and techniques to created induced pluripotent stem cells, controversy has crept in, both related to abortion politics and to human cloning. Additionally, efforts to market treatments based on transplant of stored umbilical cord blood have proven controversial.

Features of Multipotent Mesenchymal Stromal Cells

Mesenchymal stem cells, or MSCs, are multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells) [25]. This phenomenon has been documented in specific cells and tissues in living animals and their counterparts growing in tissue culture.

Because the cells, called MSCs by many labs today, can encompass multipotent cells derived from other non-marrow tissues, such as placenta, umbilical cord blood, adipose tissue, adult muscle, corneal stroma or the dental pulp of deciduous baby teeth, yet do not have the capacity to reconstitute an entire organ, the term multipotent stromal cell has been proposed as a better replacement [23, 190].

The youngest, most primitive MSCs can be obtained from the umbilical cord tissue, namely Wharton's jelly and the umbilical cord blood. However the MSCs are found in much higher concentration in the Wharton’s jelly compared to the umbilical cord blood, which is a rich source of hematopoietic stem cells. The umbilical cord is easily obtained after the birth of the newborn, is normally thrown away, and poses no risk for collection. The umbilical cord MSCs have more primitive properties than other adult MSCs obtained later in life, which might make them a useful source of MSCs for clinical applications.

An extremely rich source for MSCs is the developing tooth bud of the mandibular third molar. While considered multipotent, they may prove to be pluripotent. The stem cells eventually

form enamel, dentin, blood vessels, dental pulp, and nervous tissues, including a minimum of 29 different unique end organs. Because of extreme ease in collection at 8–10 years of age before calcification, and minimal to no morbidity, they will probably constitute a major source for personal banking, research, and multiple therapies. These stem cells have been shown capable of producing hepatocytes.

Additionally, amniotic fluid has been shown to be a rich source of stem cells. As many as 1 in 100 cells collected during amniocentesis, has been shown to be a pluripotent MSCs.

Adipose tissue is one of the richest sources of MSCs. There are more than 500 times more stem cells in 1 gram of fat than in 1 gram of aspirated bone marrow. Adipose stem cells are actively being researched in clinical trials for treatment of a variety of diseases.

The presence of MSCs in peripheral blood has been controversial. However, a few groups have successfully isolated MSCs from human peripheral blood and been able to expand them in culture [38].

Mesenchymal stem cells are characterized morphologically by a small cell body with a few cell processes that are long and thin (Figure 5). The cell body contains a large, round nucleus with a prominent nucleolus, which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. The remainder of the cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. The cells, which are long and thin, are widely dispersed and the adjacent extracellular matrix is populated by a few reticular fibrils but is devoid of the other types of collagen fibrils [24].

MSCs have a great capacity for self-renewal while maintaining their multipotency. The standard test to confirm multipotency is differentiation of the cells into osteoblasts, adipocytes, and chondrocytes as well as myocytes and neurons. MSCs have been seen to even differentiate into neuron-like cells, but there is lingering doubt whether the MSC-derived neurons are functional [59, 84]. The degree to which the culture will differentiate varies among individuals and how differentiation is induced, e.g., chemical vs. mechanical; and it is not clear whether this variation is due to a different amount of "true" progenitor cells in the culture or variable differentiation capacities of individuals' progenitors [53]. The capacity of cells to proliferate and differentiate is known to decrease with the age of the donor, as well as the time in culture.

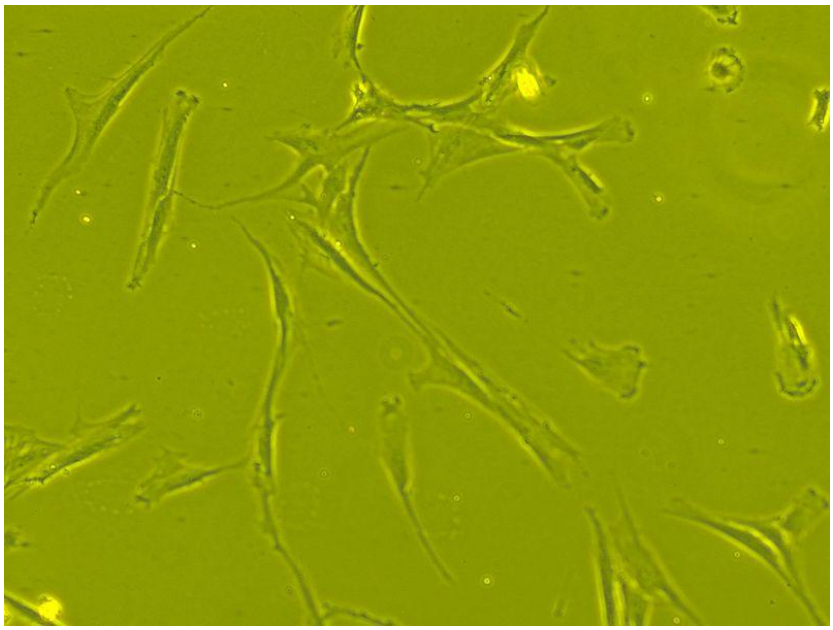

Figure 5 - Human bone marrow derived mesenchymal stem cell showing fibroblast like morphology seen under phase contrast microscope

Clinical Applications of Mesenchymal Stem Cells

Genetically modified MSCs are under research as novel cell-based transgenic cell therapy to induce or enhance production of biomolecules useful in several fields of medicine and particularly in cancer [159].

MSCs may act as a vehicle for anti-tumor molecules to counteract tumour growth. In many studies, researchers inserted therapeutic genes into MSCs and delivered them as a vehicle of therapeutic cytokines like interferon-β (IFN-β) [131], and IL-2 [132]; as a vehicle of some enzymes like cytosine deaminase expressing AT-MSC [13]; used them as a receptor ligands carrier like tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) [39, 107]; and as a carrier of oncolytic viruses [94, 171].

Virotherapy specifically kills tumor cells by targeting and infecting malignant tumor cells with oncolytic viruses. Adenoviruses are the most suitable virus vectors for developing effective oncolytic therapies for brain tumors, but they have some limitations, for example, lack of enough viral distribution in tumor site [94]. Oncolytic adenoviral vector loaded MSCs deliver therapeutic genes to elicit a significant antitumor response in animal models of intracranial glioma, medulloblastoma, melanoma, brain metastasis, disseminated neuroblastoma and breast cancer lung metastasis [1].

Adult cardiomyocytes have no ability to regenerate and after myocardial infarction (MI) damaged myocardium is remodeled by fibrous scar. As a result of this, ventricle loses its

contractile strength and injured cardiac tissue leads to ventricular dysfunction and arrhythmias. After MI, all of these outcomes cause progression to heart failure in time. Stem cell-based therapies are recently under research in laboratories and clinical trials [74]. In recent studies, different type of stem cells, such as bone marrow-derived mononuclear cells, MSCs, cardiac stem cells, and cell combinations, have been administered directly intramyocardial (trans-epicardially or trans-endocardially) or intravenously to restore and regenerate cardiac tissue [19, 163].

Mesenchymal stem cells is applied as a new promising treatment for heart failure and MSCs have some advantages due to their ability to trans-differentiate into functional cardiomyocytes, multilineage plasticity, paracrine effects and ease of accessibility regarding to the other stem cells [145].

Recent clinical trials have shown benefits and regenerative effects of intramyocardial injections of bone marrow–derived MSCs to improve regional function with improvements in ejection fraction and reversing scar formation.

Acute stress, sepsis, inflammation can also cause irreversible lose of cardiomyoctyces. Rogers et al. applied acute stress protocols; incubations with either endotoxin, lipopolysaccharide (LPS) or toxic cytokine in the cultures of neonatal mouse ventricular cardiac myocytes (nMCM) to induce experimental cardiomyocytyces damage. In this study, damaged cardiomyocytes were co-cultured with MSCs to understand cellular and molecular mechanisms of the effects of MSCs on damaged cardiomyocytes. Their results revealed that MSCs protected nMCM from the damaging effects of either LPS or IL-1β. MSCs showed also reparative effects on cardiac cells through molecular reprogramming of the cardiac myocytes [154]. In brief, MSCs are widely under research for ischemic cardiomyopathy, heart failure and cardiac injury as a promising cell-based therapy and whole animal studies and early human clinical trials demonstrate that hMSCs can improve cardiac functions and stimulate cardiac repair and regeneration after cardiac injury.

Liver failure is a serious liver disease with massive necrosis of liver cells and liver transplantation is the only crucial therapy for these patients. However, lack of donors, side effects for donors, surgical complications, rejection, and high cost are serious problems. Stem cell therapies are promising and widely interesting research area for liver failure, fulminant hepatitis, autoimmunity and metabolic liver diseases [96]. There are some clinical trials about MSCs treatment in liver diseases. Recent studies show evidences that MSCs may be a key

element for hepatic regeneration and restoring hepatic functions after hepatic diseases, cirrhosis or hepatectomy.

Mesenchymal stem cell therapy is widely under research for treatment of renal injury, and kidney diseases such as diabetic nephropathy, lupus nephritis, and acute kidney injury. MSCs are also a promising therapeutic candidate for graft versus host disease after kidney transplantation with their ability to modulate immune responses by secretion of various growth factors and cytokines [41, 98, 103]. IL-6, IL-10, HGF, prostaglandin E2, transforming growth factor-β(TGF-β), and IFN-γ are secreted by MSCs. MSCs inhibit T-cell proliferation, monocyte differentiation and modulate B-cell functions with secreted cytokines and growth factors [134]. Due to their ability to regulate immunomodulation, MSCs have been studied in many clinical trials for treatment of graft versus host disease [97]. MSCs migrate to injured kidney and gene modified MSCs act as a vehicle for cell-based transgenic cell therapy to induce or enhance production of useful biomolecules [72, 202].

The ability of MSCs to differentiate into osteoblasts, tenocytes, and chondrocytes has attracted interest for their use in orthopedic settings. First, MSCs have been shown to be beneficial in treating bone disorders, such as osteogenesis imperfecta (OI) and hypophosphatasia. Osteogenesis imperfecta is characterized by skeletal fragility and connective tissue alterations caused by alteration of type I collagen production by osteoblasts. In a study, pediatric patients with OI underwent allogenic hematopoietic stem cell transplantation (HSCT) and MSC and the transplanted bone marrow cells engrafted and generated functional osteoblasts leading to improvement in bone structure and function [79]. Hypophosphatasia is a genetic disorder of mesenchymal origin with mutation in tissue nonspecific alkaline phosphatase. Although the numbers of clinical studies are limited, pediatric patients who received BMT showed significant clinical improvements [31, 193].

Modalities for Bone Regeneration

Unlike other tissues, the bone can regenerate and repair itself: in many instances, bone injuries and fractures heal without scar formation [49, 133]. Nevertheless, in pathological fractures or large and massive bone defects, bone healing and repair fail. Insufficient blood supply, infection of the bone or the surrounding tissues, and systemic diseases can negatively influence bone healing, resulting in delayed unions or non-unions [12, 17]. A bone graft is defined as an implanted material that promotes bone healing alone or in combination with

other materials, through osteogenesis, osteoinduction, and osteoconduction, in combination or alone [52]. The materials used in bone grafting can be divided into several major categories, including autografts, allografts, and xenografts. Synthetic and biologically based, tissue-engineered biomaterials and combinations of these substitutes are other options [45]. Autografts are the 'gold standard' in reconstructing small bone defects and have strong osteogenic characteristics relevant to bone healing, modeling, and remodeling [10]. Allografts are an alternative option with major limitations associated with rejection, transmission of diseases, and cost. Allografts have lower incorporating properties with the host healing tissues as compared with autografts [201]. Xenografts, in addition to the disadvantages of allografts, carry the risks of transmission of zoonotic diseases, and rejection of the graft is more likely and aggressive. Given these problems, tissue engineering has been introduced in the last decade. Tissue engineering involves using relevant scaffolds, introducing appropriate growth factors and cells, and, more recently, the use of stem cells. Using tissue engineering techniques, it is possible to design new scaffolds and tissue grafts aiming to decrease the disadvantages of traditional grafts and improve graft incorporation, osteogenicity, osteoconductivity, and osteoinductivity [45]. An ideal bone graft material should have osteogenesis, osteoinductivity, osteoconductivity, and osseointegration characteristics [3, 133, 141].

Osteogenesis is the capacity to produce new bone by the osteoblasts by differentiation of osteoprogenitor cells either present in the recipient bone or coming from the graft material. This property is mainly present in autogenous grafts as compared with allografts and xenografts, because the cellular structures of the allografts and xenografts have low viability after implantation [66, 89]. Osteoinduction is the capability of the graft materials to induce formation of the bone-forming cells via differentiation of MSCs of the surrounding host tissues to produce osteoprogenitor cells followed by development of osteoblasts. Such ability has been discovered in growth factors including bone morphogenetic proteins (BMPs) such as BMP-2 and BMP-7, transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) [3, 89, 133, 141]. Osteoconduction is a characteristic whereby the graft acts as a permanent and resorbable scaffold, mechanically supporting ingrowth of vessels and new bone from the borders of the defect into and onto its surfaces. This characteristic initiates or induces new bone formation [3, 44, 141]. Finally, osseointegration is the ability to bind to the surrounding bone without an intervening layer of fibrous tissue, allowing incorporation of the graft at the host site [3]. All

bone grafts and bone-graft substitute materials can be described by these processes [66]. Among all types of bone grafts, only autografts possess all the above features. Allografts and xenografts exhibit only two or three of the four features of an ideal bone graft material (osseointegration, osteoconduction, and perhaps osteoinduction) and lack osteogenic properties [160].

Bone grafts that are harvested from one site and implanted into another site within the same individual are termed autografts, autologous, or autogenous bone grafts [204]. They may be cancellous or cortical (non-vascularized or vascularized) bone, and in some instances a combination of both, 'cortico-cancellous grafts'. Fresh autografts contain surviving cells and osteoinductive proteins such as BMP-2, BMP-7, FGF, IGF, and PDGF [26]. From a biological point of view, they are the best material available, since they totally lack immunogenicity. They retain their viability immediately after transplantation, and the lack of immunogenicity enhances the chances of graft incorporation into the host site [83]. Furthermore, the osteogenic, osteoinductive, and osteoconductive properties of fresh autografts are optimal, given the presence of MSCs, osteoprogenitor cells, osteogenic cells, and growth factors [89]. Autografts have no associated risk of viral transmission; moreover, they offer structural support to implanted devices and, ultimately, become mechanically efficient structures as they are incorporated into the surrounding bone through creeping substitution [66]. The main drawback is that autografts must be harvested from another body site, which implies additional surgery with a higher chance of donor site pain, morbidity, and complications. If massive grafting is needed, adequate amounts of autograft may not be available, and other bone graft materials have to be considered.

Allografts are harvested from one individual and implanted into another individual of the same species. Given the limitations associated with harvesting autografts, allografts have been applied clinically and experimentally as a common alternative to autografts [130]. Allografts are used in both morselized and structural forms and are provided as cortical, cancellous, or cortico-cancellous grafts and in various shapes such as powder, cortical chips, and cancellous cubes. They also can be processed as mineralized or demineralized, fresh, fresh-frozen, or freeze-dried forms. Allografts can be obtained from cadavers or living donors. The cadaveric form is available as a commercial product. Allografts obtained from fresh cadavers with preservation of their cellular and organic content are minimally processed [22]. The major advantages of allografts are their ready availability in various shapes and sizes, avoidance of the need to sacrifice host tissues, and no challenges of donor site morbidity. Allografts have

variable osteoinductive and osteoconductive properties but lack viable cells and, therefore, have lower osteogenic potential than autografts [204].

Another alternative to autogenous bone grafts are xenografts, also known as heterologous or xenogenic grafts [43]. Xenografts are harvested from one individual and transplanted into another individual of a different species. The common available xenografts are derived from coral (Biocoral®, natural coral; Biocoral Inc, Wilmington, New Castle, DE, USA), porcine, and bovine sources [43]. Xenogenous bone grafts are a theoretically unlimited supply of available material if they could be processed to be safe for transplantation in humans. A major concern with bovine-derived products is the potential transmission of zoonotic diseases and prion infections such as bovine spongiform encephalitis (BSE) [138]. Xenografts, similar to allograft, lose their osteogenic and partly osteoinductive properties during the processing to counteract their antigenic properties and prevent transmission of infection. Xenografts produce poor clinical outcome; however, new insights have been presented.

Tissue engineering is the 'final' option in managing bone loss. Tissue engineering can involve the use of scaffolds, healing promotive factors (e.g., growth factors), and stem cells. Tissue engineering is defined as 'a process that affects the structure and architecture of any viable and non-viable tissue with the aim to increase the effectiveness of the construct in biologic environments'. Therefore, all the non-fresh grafts which are processed for acellularization belong to the tissue engineering category. In fact, acellularization is the basic tissue engineering technology described for allograft and xenografts [184]. This method of tissue engineering has been used for many years to decrease the antigenicity of the viable grafts [184].

Scaffolds are the most important issue in tissue engineering and could be divided into two main categories including biological (natural or organic) and synthetic (artificial) materials [141]. The former are natural polymers such as collagen type I or DBM [2]. Porous metals, bioactive glasses, synthetic polymers such as polylactic acid (PLA) and polyglycolic acid (PGA), and calcium phosphate ceramics such as hydroxyapatite (HA) and tricalcium phosphates (TCP) are examples of synthetic materials [183].

Natural- or biologic-based materials are taken from biologic-based tissues, and xenografts may be the best source for these later products. The advantages of natural-based scaffolds are that they have significantly superior biocompatibility, biodegradability, regenerative characteristics (e.g., osteoinduction, osteoconduction, osteogenesis, and osteointegration) than

those of synthetic materials, but their immunological behavior is variable in different species and is also related to the type of application [90].

Several in vitro and in vivo researches tried to optimize synthetic-based, tissue-engineered scaffolds in order to be useful in bone regenerative medicine. A single-walled carbon nanotube (SWCNT) and polylactic-co-glycolic acid (PLGA) composite has been developed recently. After seeding with human MSCs and osteoblasts, the composite imparted beneficial cellular growth capability and gene expression, and mineralization abilities were well established suggesting its potential application in bone regeneration [71]. As another strategy, a combination of different polymers has been tried to increase the cell cytocompatibility of the synthetic-based scaffolds. Poly(l-lactide) and poly(caprolactone triol) are some examples. Using such combination, new membranes promoted the rat osteoblastic cell behavior in vitro (e.g., migration, attachment, proliferation, and matrix production) [121]. Surface modification and coating is another strategy to enhance bioactivity of the synthetic scaffolds. Silica nanoparticles have been applied onto the fiber surface of an interbonded three-dimensional polycaprolactone (PCL) fibrous tissue scaffold. The nanoparticle layer was found to improve the fiber wettability and surface roughness. Thus, it enhanced osteoblast attachment, proliferation, and alkaline phosphatase activities [180].

Despite many beneficial characteristics of synthetic materials in bone healing and regeneration, their biocompatibility, biodegradability, and regenerative properties are still suboptimal compared to natural-based scaffolds. Therefore, many attempts have been made to combine synthetic with natural materials. Recently, poly(D,L-lactideco- glycolide) has been combined with a naturally bioceramic hybrid material, nanonized pearl powder, as an osteoinductive material: the scaffold was able to influence osteoblast behavior in vitro [200]. The benefits associated with polyhydroxyalkanoates (PHA) in tissue engineering include high immunotolerance, low toxicity, and biodegradability.

Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), a molecule from the PHA family of biopolymers, shares these features. Collagen has been used with PHA to increase the biocompatibility of the scaffold and to support cell proliferation and osteogenic differentiation in vitro [108].

There is an increasing demand for an injectable cell coupled three-dimensional (3D) scaffold to be used as bone fracture augmentation material. To address this demand, a novel injectable osteogenic scaffold called PN-COL was developed, using cells, a natural polymer (collagen

type I), and a synthetic polymer (PCL). The injectable nanofibrous PN-COL is produced by interspersing PCL nanofibers within pre-osteoblast cell embedded collagen type I. This simple yet novel and powerful approach provides a great benefit as an injectable bone scaffold over other non-living bone fracture stabilization polymers, such as polymethylmethacrylate and calcium content resin-based materials. The advantages of injectability and the biomimicry of collagen were coupled with the structural support of PCL nanofibers to create cellencapsulated Injectable 3D bone scaffolds with intricate porous internal architecture and high osteoconductivity. The effects of PCL nanofiber inclusion within the cellencapsulated collagen matrix have been evaluated for scaffold size retention and osteocompatibility, as well as for MC3T3-E1 cells osteogenic activity. At structural analysis, this novel bioactive material was chemically stable enough in an aqueous solution for extended periods without using crosslinking reagents, but it is also viscous enough to be injected through a syringe needle. Data from long-term in vitro proliferation and differentiation suggests that PN-COL scaffolds promote osteoblast proliferation, phenotype expression, and formation of mineralized matrix [15].

Healing promotive factors such as growth factors have been extensively used to treat bony defects and for osteoinduction. Some growth factors such as vascular endothelial growth factor (VEGF), TGF-β, PDGF, and BMPs such as BMP-2, BMP-7, and IGF are present in the healthy bone matrix and are expressed during bone healing [173]. These factors can regulate vascularization and induce proliferation and differentiation of the osteoblasts and their precursors, so they can be useful in improving the healing processes.

Bone morphogenetic protein-2 (BMP-2) is a potent osteoinductive cytokine that plays a critical role during bone regeneration and repair. In the extracellular environment, sulfated polysaccharides anchored covalently to glycoproteins such as syndecan and also non-covalently to fibronectin fibers have been shown to bind to BMP-2 through a heparin-binding domain and regulate its bioactivity [99]. The supramolecular peptide amphiphile nanofibers, which integrate the biological role of syndecan and fibronectin, have been controlled and designed to form as a network within the pores of an absorbable collagen scaffold. The hybrid biomaterial enhanced significantly bone regeneration in a rat critical size femoral defect model using BMP-2 in amounts that are one order of magnitude lower than that required for healing in this animal model. Although many reports confirmed the beneficial effects of BMP on bone regeneration and quality, some others showed their ineffectiveness on regeneration of nonweight bearing bone healing. In an investigation, the biomechanical properties of calvarial

bone regenerated with derivations of a commercially available rhBMP-2-based system were evaluated. Standardized calvarial defects were produced in 23 adult male canines. These defects were treated with rhBMP-2 on one of the several carriers. After 24 weeks, the biomechanical properties of the rhBMP-2-generated bone were compared to those of the controls with a modified punch-out test. They concluded that rhBMP-2-generated calvarial bone is significantly less protective against trauma than native bone at 6 months. TGF-β1 is crucial in the development, induction, and repair of bone. The effect of local application of a graft DBM along with TGF-β1 in a model of open osteotomy induced experimentally in dogs has been investigated [165]. An open osteotomy of the tibia was produced in young male dogs. On the fifth week, there was an improvement and restoration of bone architecture in animals treated with a graft containing TGF-β1 (5 ng/mL) compared with the control and graft groups, as evidenced by early formation of wide callus and bone regeneration. In addition, local application of TGF-β1 led to an increase in collagen and proteolytic activity. More immunopositive osteoclast and mesenchymal cells were found in the bone tissue from animals treated with TGF- β1 compared with the control group.

Alendronic acid or alendronate sodium, sold as Fosamax by Merck (Whitehouse Station, NJ, USA), is a bisphosphonate drug for osteoporosis and several other bone diseases. It is marketed alone as well as in combination with vitamin D. On February 6, 2008, the US FDA approved the first generic versions of alendronate, which were marketed by Barr Pharmaceuticals (Montvale, NJ, USA) and Teva Pharmaceuticals (Horsham Road North Wales, PA, USA) [18]. Mathijssen et al. investigated the role of several materials and drugs on bone healing [117]. Twenty-five goats received eight bone conduction chambers in the cortical bone of the proximal medial tibia. Five concentrations of alendronate (0, 0.5, 1, 2, and 10 mg/mL) were tested in combination with allograft bone and supplemented with cefazolin (200 μg/mL). Allograft not supplemented with alendronate and cefazolin served as control. In addition, allograft mixed with DBM, with and without alendronate, was tested. After 12 weeks, a dose–response relationship for local application of alendronate was detected. Local application of cefazolin had no effect on bone remodeling.

Platelet-rich plasma (PRP) is a simple way of delivering growth factors [139]. The combination of human PRP with HA may be a promising alternative for reconstruction and regeneration of critical size defects in animal models [139]. More recently, a combination of PRP with silicon stabilized HA/TCP scaffold has been effective in rabbit calvarial defect (Skelite™; Millenium Biologix Corporation, Kingston, Ontario, Canada). Using such

strategy, significant osteoid-like matrix and new bone deposition together with higher cellularity, more abundant osteoid deposition, and more regular collagen fibers could be seen in micro-CT and histologic analysis when compared to scaffold alone. Moreover, in vitro migration assays confirmed the chemotactic effect of PRP to endothelial and osteoprogenitor cells. Addition of PRP influenced the local tissue microenvironment by providing key cryptic factors for regeneration, thereby enhancing the progenitor cell recruitment and collagen and bone matrix deposition and creating a bridging interface between the scaffold and bone [51]. Some controversies exist in the effectiveness of PRP. The effect of autologous PRP on the early phases of osteoinduction by allogenic DBM in rabbit intramuscular positions has shown that addition of PRP to DBM had a negative effect on the early phases of osteoinduction at 3 weeks [102]. Faratzis et al. investigated the effect of autologous PRP on the osteogenic potential of a biphasic synthetic graft material composed of HA/β-TCP in critical size cranial defects in rabbits [55]. Autologous PRP in addition to a biphasic HA/β-TCP synthetic graft material had no effect on bone healing after 2, 4, and 6 weeks of implantation.

The combination of stem cells with scaffolds as a polytherapy is a new option. Collagen and demineralized bone powder have been used to produce a novel scaffolds for bone tissue engineering. Human periosteum derived cells (PD cells) were cultured on this scaffold: the hybrid scaffolds exhibited greater osteoinductive potential than collagen scaffolds. The PD cells with hybrid scaffolds possessed higher ALP activity, calcium deposition, and superior behavior (e.g., attachment, differentiation, and proliferation) than those with collagen scaffolds [181].

The feasibility of applying calcined bovine bone (CBB) coated with allograft bone marrow MSC - sheet as a 3D scaffold material in bone healing has been investigated recently. The new scaffold material was implanted into osteoporosis rat cranial bone defects and critical size bone defects (8-mm diameter). The CBBBMSC-sheet combination had a stronger potential in osteogenic differentiation and mineralized formation both in vitro and in vivo than CBB-BMSC combination. Three dimensional reconstruction of micro-CT, H&E staining, and bone strength results showed that the area and volume of the newly formed bone in CBB-BMSC-sheet group was significantly higher than that of the CBB-BMSC group after 4 to 12 weeks [106].

Adipose-derived stem cells (ASCs) with multilineage differentiation capacities have been demonstrated as an alternative cell candidate for in vitro and in vivo bone regeneration. This

suggests that they may be a potential candidate to repair the bone defects. Yang et al. attempted to demonstrate the use of new biomimetic constructions of undifferentiated rabbit ASCs with fully interconnected porous β-TCP scaffolds encapsulated by collagen I hydrogel in the regeneration of a critical-sized defect of rabbit radii [199]. Critical-sized defects in the left radii of rabbits were prepared and inserted with rASCs/collagen I/β-TCP scaffold composites or collagen I/β-TCP scaffold composites. Twelve weeks after implantation, the defects were almost completely repaired as confirmed by the presence of the cortical bone and medullary cavity. In addition, a greater number of ASCs in the scaffold enhanced osteogenesis in critical-sized defects. Pourebrahim et al. compared bone regeneration of tissue-engineered bone from ASCs and autogenous bone graft in a canine maxillary alveolar cleft model [146]. The undifferentiated cells were incubated with a HA/β-TCP scaffold in specific osteogenic medium for 21 days. Four mongrel dogs were prepared by removal of two of the three incisors bilaterally and a 15-mm defect in bone was created from the crest to the nasal floor. After healing, repair was followed by a tissue-engineered bone graft from ADCs on one side and cortico-cancellous tibial autograft on the other side. Bone regeneration was evaluated by histomorphometry on days 15 and 60 after implantation. The bone formation on the autograft sides was higher than on the stem cell sides at 15 and 60 days, and 45% and 96% versus 5% and 70%, respectively.

Three-dimensional printing (3DP) is a rapid prototyping technique that can create complex 3D structures by inkjet printing of a liquid binder onto powder biomaterials for tissue engineering scaffolds. Direct fabrication of scaffolds from 3DP, however, imposes a limitation on material choices by manufacturing processes. Novel additive manufacturing processes are increasingly recognized as ideal techniques to produce 3D biodegradable structures with optimal pore size and spatial distribution, providing adequate mechanical support for tissue regeneration while shaping ingrowing tissues [32].

Gene therapy consists of transfer of genetic information to the target cells and may introduce a safe and effective strategy to induce bone healing. Gene therapy can be used for delivery of growth factors in tissue engineering. The vehicle for gene delivery can be either viral (adenovirus, retrovirus, and adeno-associated virus) or non-viral (liposomes). However, this approach has a series of limitations, including trans-infection of the target cells with the foreign genes [164]. Furthermore, an unresolved issue of gene therapy is to target the right gene at the right location in the right cells and express it for sufficiently long at the right time, while minimizing adverse reactions.

Experimental Modalities for Creating Osteonecrosis of the Femoral Head

To study the efficacy of novel therapies for the ONFH, suitable animal models of ON must be created. There is no reliable animal model of the early stages of ONFH for the evaluation of new therapeutic approaches. Since the pathogenesis of ONFH is unknown, the criteria for a valid animal model are difficult to define.

Among these methods, a single injection of high dose corticosteroids is the most commonly used technique [88, 128, 196]. Intramuscular injection of 20 mg/kg of methylprednisolone induced development of ON in femurs and humeri of 43% of the rabbits at the end of 4th week. However, there were no signs of osteonecrosis in the proximal epiphyses of femurs. In addition, after 6 weeks, there was also progressive histological evidence of revascularization, with granulation tissue, and osteoblastic repair, with appositional bone formation. Hyperlipemia, fatty liver, and intraosseous fat embolism were observed in conjunction with thrombocytopenia and hypofibrinogenemia [196].

In another study, pure ethanol was injected into the centre of the femoral head in adult Merino sheep under fluoroscopic control [111]. After 3, 6 and 12 weeks the animals were killed and the femoral heads were harvested. At the end of 3rd week, many osteocytes were necrotic. The outline of dead trabeculae was maintained. In the marrow cavities mesenchymal tissue and bone debris were present. An irregular trabecular resorption was found. At the end of 6th week, in highly damaged areas (>80% of empty lacunae), most of the marrow cavities contained fibrous tissue of high cellularity with numerous macrophages. Necrotic trabeculae underwent resorption. In these areas, scanty bone formation occurred from the fibrous tissue and no enchondral bone formation was detected. The resorptive changes were not uniform. In slightly damaged areas, trabecular resorption by osteoclasts and remodelling was detectable without filling of the marrow cavities with fibrotic tissue. Thus, there was no regular resorption line. The edges of the track were covered by layers of osteoblasts and osteoid. At the 12th week, the majority of the necrotic tissue was replaced by vital bone.

Another inductive protocols commonly used for establishing ON in rabbits is to use two injections of high-dose lipopolysaccharide (LPS) combined with subsequent three injections of high-dose methylprednisolone (H-LPS×2+H-MPS×3), which induced higher ON incidence (85%) but accompanied with high mortality of experimental animals (50%) [149]. Due to the high mortality rate, this method was modified as a single injection of low-dose LPS (L-LPS×1). This model showed a higher incidence of osteonecrotic lesion (77%) and lower

mortality (11%) in rabbits as compared with the previously published protocol (H-LPS×2+H-MPS×3) due to the avoidance of severe LPS-induced shock by lowering the given LPS dose. Of the rabbits who received 10 μg/kg LPS and three injections of 20 mg/kg of methylprednisolone with an interval of 24 hours, 93% developed ON 6 weeks after last injection of methylprednisolone. In a location-specific histopathological examination for the proximal femora, 86% were found having developed ON in the metaphysis while 29% developed ON in the proximal epiphysis. Destructive repair characterized with either limited repair with appositional bone formation around necrotic bone or granulation tissue creep linked to the resorbing necrotic bone was observed in several samples.

In one study, the use of microwave heating to create osteonecrosis of the femoral head was investigated [104]. In this study, a hole was drilled from subcapital to the centre of both femoral heads of the rabbits and a microwave antenna was set into the centre of the femoral head through the drill track to apply heat. By increasing the amount of heat applied, this technique successfully induced osteonecrosis in the femoral head beginning from the 2nd week. Moreover, the repair process stopped at the 8th week whereas the bone still continued to undergo necrosis which rendered this technique suitable to study the effects of treatment on the late stages of osteonecrosis.

Other techniques used to create osteonecrosis in femoral head are refrigeration using liquid nitrogen applied directly to the femoral head, cutting of the ligamentum teres and obstruction of the blood flow to the femoral head using cerclage wire, cauterization of the extraperiosteal femoral neck vessels [81, 172].

Aim of the Thesis

The osteonecrosis of the femoral head is a relatively common problem. The proper etiopathogenesis is still unclear which makes this disease unpredictable. Since it can be encountered in young population, its effects on the patients' life quality and economical status can be worrisome. Novel joint preserving therapies are being developed every year. However, the literature lacks a proper model for the induction of osteonecrosis in the femoral head in animals. This thesis addresses two pre-clinical aspects regarding the osteonecrosis of femoral head. The first is to study the effectiveness of an animal model for the induction of osteonecrosis of the femoral in rabbits. The onset of the necrotic changes and the reparative process were studied.

The second one was to show the effect of stem cell therapy for the osteonecrosis of the femoral head. For this, the MSCs were harvested from the bone marrow of the rabbits and expanded. Then they were injected directly into the avascular zone of the femurs. The time dependent changes in the femoral heads were documented histologically.

This thesis aimed to introduce an animal model for the induction of osteonecrosis in the femoral head and demonstrate the effect of stem cell therapy for this disease.

CHAPTER 2

MATERIALS AND METHOD

Animals

Three months-old New Zealand female rabbits (Harlan Laboratories Srl, San Pietro al Natisone, Italy) weighing 3.0 Kg (n=16) were operated at the interdepartmental animal facility of the University of Modena and Reggio Emilia. The study protocol was approved by the local ethical animal Committee and by the Italian Ministry of Health. Six Animals underwent to the ONFH induction and were sacrificed at 2, 4 and 6 weeks after damage in order to assess how long the ON event developed in the femoral head. Based on these pilot study results, we will define the timing for the development of ON event. Ten rabbits were sacrificed to analyze the effects of stem cells therapy for the osteonecrosis of the femoral head.

Surgical Procedures, BM-MSC Isolation and Expansion

Induction of Femoral Head Osteonecrosis

Each rabbit was sedated with intramuscular injection of 1mg/kg midazolam and anesthetized with 3% Sevorane (Abbott S.r.l., Latina, Italy) using a vaporizer through facial mask. The fur on the left thigh is completely removed. The surgical site is disinfected using baticon. A lateral skin incision was performed over the great trochanter. The incision was extended distally 2 cm over the lateral aspect of femur and 2 cm proximally making a slight curve posteriorly. After incising the subcutaneous tissue beneath the skin incision, the tensor fascia lata was split open. The gluteus medius muscle was detached from the great trochanter and elevated anteriorly. The anterior aspect of hip capsule was made visible and a “T” cut was done on the capsule. Electrocoagulation was performed all around the femoral neck extending from the great trochanter to the cartilage on the femoral head that was left untouched (Figure 6). A lateral traction to the proximal femur was performed in order to dislocate the femoral head and to see the ligamentum teres which was then cut to assure that no extraosseous circulation to the femoral head remained. The lateral traction was discontinued and the hip was reduced. The layers were appropriately sutured. Isoflurane is discontinued and the rabbit was wakened using %100 oxygen (3 l/min). The rabbit was then put in its cage and was allowed to walk freely in its cage. The same procedure was performed on the contralateral hip. Intramuscular injection of 20 mg/kg Methylprednisolone was performed. Intramuscular injection of antibiotic (BAYTRIL, Bayer HealthCare LLC Animal Health Division, Pittsburgh, USA) was given as prophylaxis. Isoflurane was discontinued and the rabbit was

wakened using %100 oxygen (3 l/min). The rabbit was then put in its cage and was allowed to walk freely in its cage.

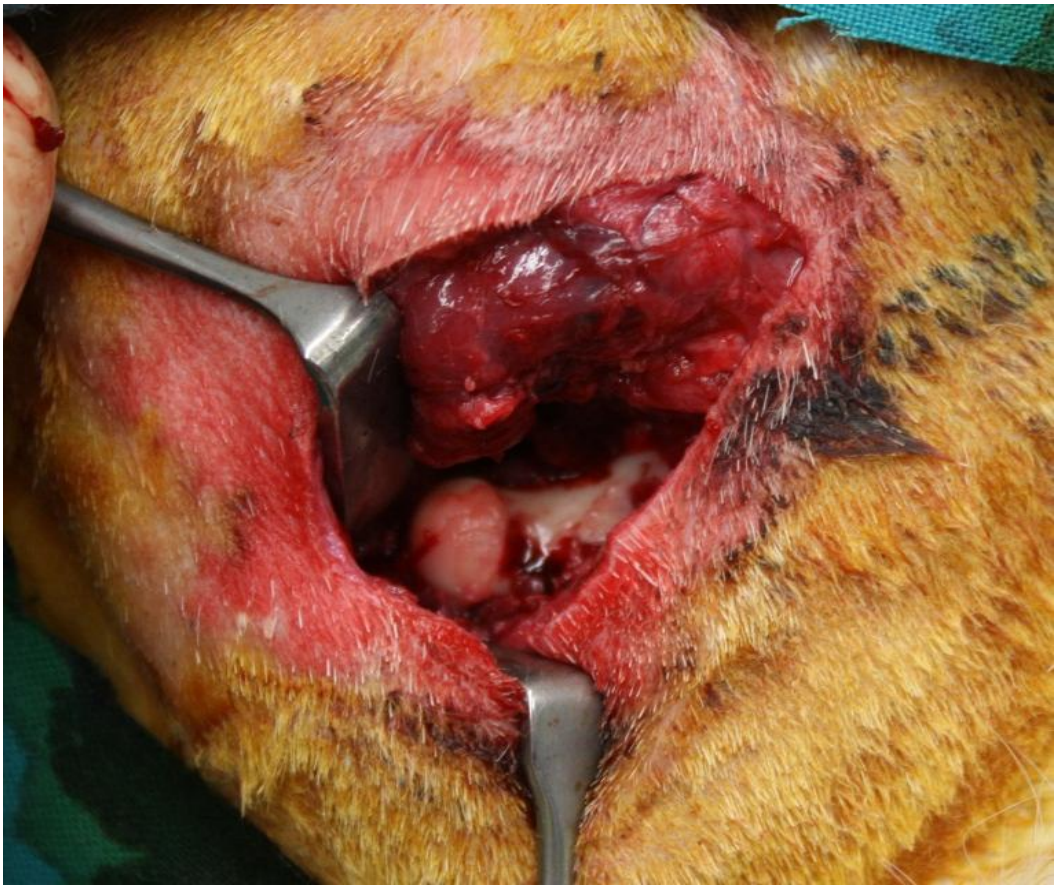

Figure 6 - After skin incision, the subcutaneous and muscle layers were opened appropriately. The femoral neck vessels were cauterized. Care was given to preserve cartilage

Bone Marrow Aspiration

For the bone regeneration study, ten 3 months-old New Zealand female rabbits underwent to the harvest of bone marrow (BM) for MSC isolation in general anesthesia. The rabbits are prepared for the procedure performed as described before. After preparatory procedures and skin sanitization, a 1 cm skin incision was performed on the lateral side of right lateral femoral condyle. The bone was penetrated using an 18 G T-type BM aspiration needle. The tip of the needle was advanced in the medullary canal while a negative pressure was applied using a 10 ml syringe pre-washed with Heparin (Hospira Italia, srl, Napoli, Italia). After aspirating 2 mL of BM, the needle was drawn back and the skin sutured closed (Figure 7).

Isoflurane is discontinued and the rabbit was wakened using %100 oxygen (3 l/min). The rabbit was then put in its cage and was allowed to walk freely in its cage.

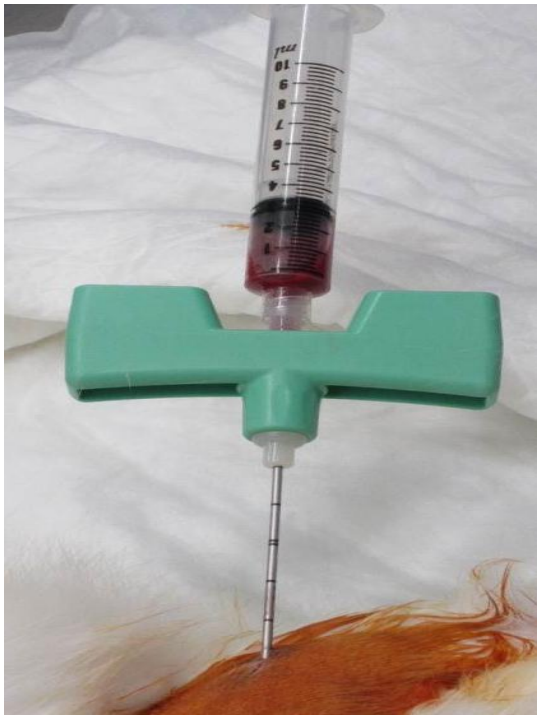

Figure 7 - Approximately 2 ml of bone marrow was aspirated from the supracondylar region of right femur

BM-MSC Isolation and Expansion

Bone marrow samples taken from femoral condyle region with a 10ml Leur-Lock syringe containing 200 µl Heparin (25000UI/ml) were diluted 1:1 in phosphate buffered saline PBS (PAA Laboratories GmbH, Pasching, Austria) and passed 20 times through a sterile 10-mL syringe (Becton Dickinson Plastipak, Drogheda, Ireland) with a 19-G needle. Samples are then treated with a lysis buffer solution.

Briefly, 0,15M ammonium chloride, 0,009M potassium bicarbonate and 0,01mM ethylenediaminetetraacetic acid (EDTA) (Euroclone) were dissolved in 1l dd water and mixed in a ration 2:1 with initial volume of BM and incubate at 37°C by gentle agitation. The samples are then centrifuged at 306 g for 10 minutes, counted by 0.4% trypan blue exclusion (Biochrom AG, Berlin, Germany) and inoculated in culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) at the density of 1.000.000 cells/cm^2 with Dulbecco's Minimum Essential Medium (DMEM) Alpha without nucleosides (Gibco® Invitrogen, UK), supplemented with 8% Platelet lysate (PL), 1% L-Glutamine (Gibco® Invitrogen, Belgium),

1 UI/ml heparin (Sigma-Aldrich, USA) and 10 μg/mL ciprofloxacin (HIKMA, Portugal). The cells were kept in incubators with a controlled atmosphere (5% CO2, 37 °C) and the medium was replaced every 2–3 days, discarding non-adherent cells.

Nine days after seeding, fibroblastic colony-forming units (CFU-F) were detectable and counted by invert microscope (Axio Observer A1 with Plan-NeoFluar 10x objective). Clones of more than 50 cells were considered to be colonies. Once 80–90% confluence was reached the BM-MSCs were detached using trypsin 0.05% and EDTA 0.02% (PAA Laboratories, Austria), were counted and seeded at 6000/cm^2. Extended cultures were also protracted until reaching passage 4. At each passage, cells were counted using 0.4% trypan blue exclusion, to evaluate viable cell expansion. When cells reach passage 4, doubling cells (DC) were calculated according to the formula: DC = Log10(Pi+1/Pi) /(log210) where Pi means number of cells in each passage in vitro.

Fluorescence-activated cell sorting analyses (FACS)

Culture expanded BM-MSC samples were detached from plastic support with trypsin and EDTA, were counted and aliquoted in FACS analysis polypropylene tubes (0,5 – 1 x 10^6/tube) (Falcon). BM-MSC were subsequently incubated in the blocking buffer (100 μl each 0.5 – 1 x 10^6 cells) containing DMEM, 10 % PBS, 0,1 M Sodium Azide and human immunoglobulin G (Sigma) and incubated for 20 minutes on ice. After PBS wash, cells were stained in ice for 30 minutes with primary monoclonal antibodies (MoAb) in PBS with 0,1% Bovine Serum Albumin (BSA, Sigma) and then with secondary antibody, where applicable. BM-MSCs ($1x10^6$) were labeled with the following monoclonal antibodies: unconjugated anti-rabbit CD45, unconjugated anti-human CD29, (all from Serotec, Milan, Italy). The appropriate isotype controls (BD Pharmigen and BD) and allophycocynanin (APC) conjugated secondary antibody (BD) were introduced where applicable. The rBM-MSC were analysed by FACSAria III flow cytometer equipped by Diva software (Becton-Dickinson) and 10.000 events were acquired.

Differentiation assays

Cultured BM-MSCs were tested for their ability to differentiate into the main mesodermal lineages. Briefly, BM-MSC were seeded in bone induction medium with alpha MEM (aMEM) (Life technologies) containing 8% pridoxal phosphate (PLP), 1 % P/S and glutamine (2 mM; Euroclone, Padmington, UK) supplemented with dexamethasone (10 nM), L-ascorbic

acid-2-phosphate (0.1 mM), beta-glycerol phosphate (2 mM) (all Sigma) and bone morphogenic protein BMP2 (100 ng/ml; PeproTech, Rocky Hill, NJ, USA). After 2 weeks of induction, differentiated BM-MSCs and controls were stained with ddH_2O 1 % silver nitrate (Sigma). Dark deposits were quantified by Image J software. BM-MSCs were induced towards adipogenic lineage using DMEM with addition of 1 % P/S, 10 % rabbit serum (Euroclone) and 5 % horse serum (Hyclone) supplemented with dexamethasone (1 mM), indomethacin (60 mM), rh-insulin (10 mM) and isobutylmethylxantine (0,5 mM; all from Sigma). BM-MSCs were maintained in differentiation media for 10 days and visualization of adipocyte differentiation was performed with Oil Red O (Sigma). After induction differentiated cells and controls were visualized by microscopical observations (Axio Observer A1 with Axiocam MRC5 color camera and Axiovision 4.82 software; all Zeiss).

Samples Preparation for Engraftment

Once BM-MSC reached P3, cells were detached as previously described and $10x10^6$ were suspended in 500 µl of normal saline ad loaded in 1 ml tubercoline syringe (B. Braun, Germany). The volume engrafted is 100 µl corresponding to $2x10^6$ BM-MSC for the empty volume of the syringe and of the intravenous cannula used for the engraftment.

Administration of stem cells into the femoral head

The rabbits are prepared for the procedure performed as described before. A 2 cm lateral skin incision is performed over the great trochanter. Subcutaneous tissue is cut and tensor fascia lata is split open. Great trochanter and the metaphyseal region of the femur were visualized. Just 3cm distal to the tip of great trochanter, on the posterior aspect, the bone was perforated using a sharp bone perforator. A 2 mm hole was created. An IV cannula (16G) was inserted and advanced approximately 2,5cm through this hole towards the femoral head. At this point fluoroscopic control is performed to ensure the right positioning of the needle in the subcartilage part of the femoral head. Before the injection of stem cells, the hole was sealed using bone wax while the needle was still inside the bone. At the point one assistant injected the stem cells through the IV cannula while the second assistant applied pressure on the bone wax so that as soon as the needle was drawn back after injection, the hole was sealed by bone wax (Figures 8, 9, 10). The layers were properly closed. Isoflurane was discontinued and the rabbit was wakened using %100 oxygen (3 l/min). The rabbit was then put in its cage and was allowed to walk freely in its cage.

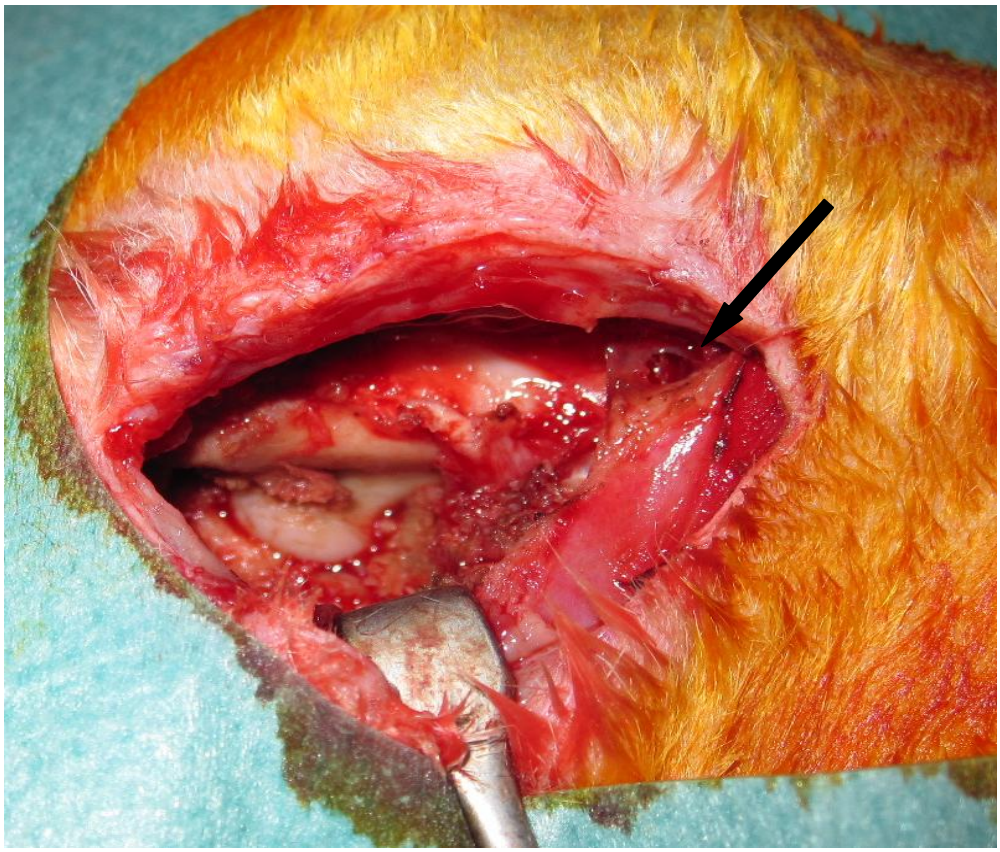

Figure 8 - After the lateral side of the proximal femur was exposed, a 2 mm hole was created (arrow) for the insertion of the cannule

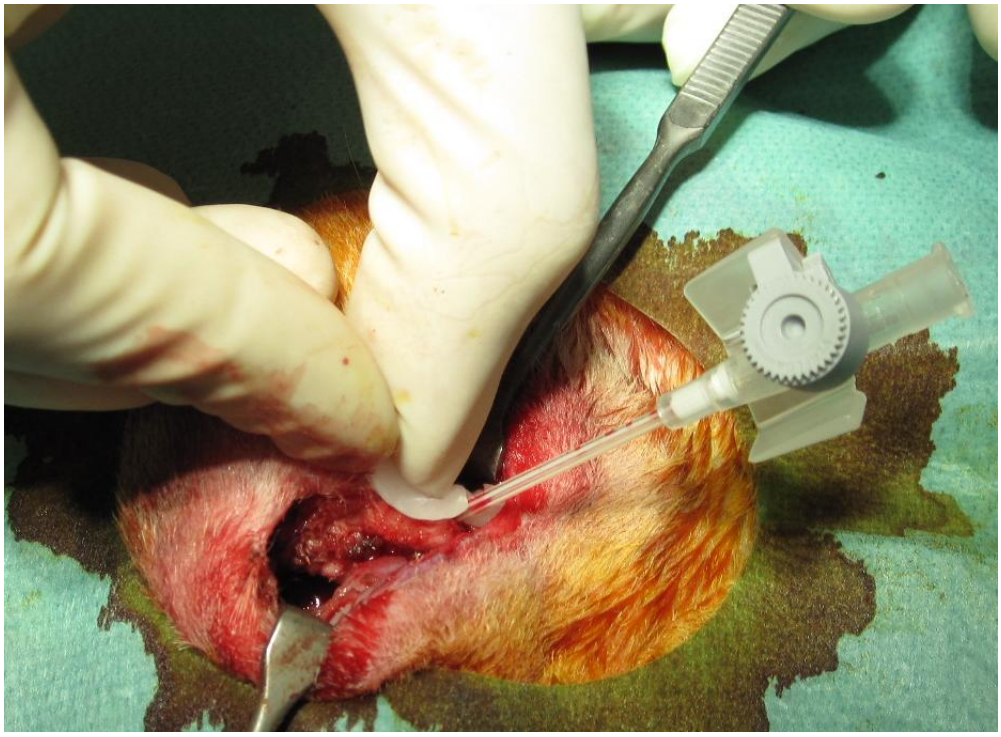

Figure 9 - The canule was inserted into the hole and advanced until the subchondral bone did not permit any further advancement. The periphery of the cannula was sealed using bone wax

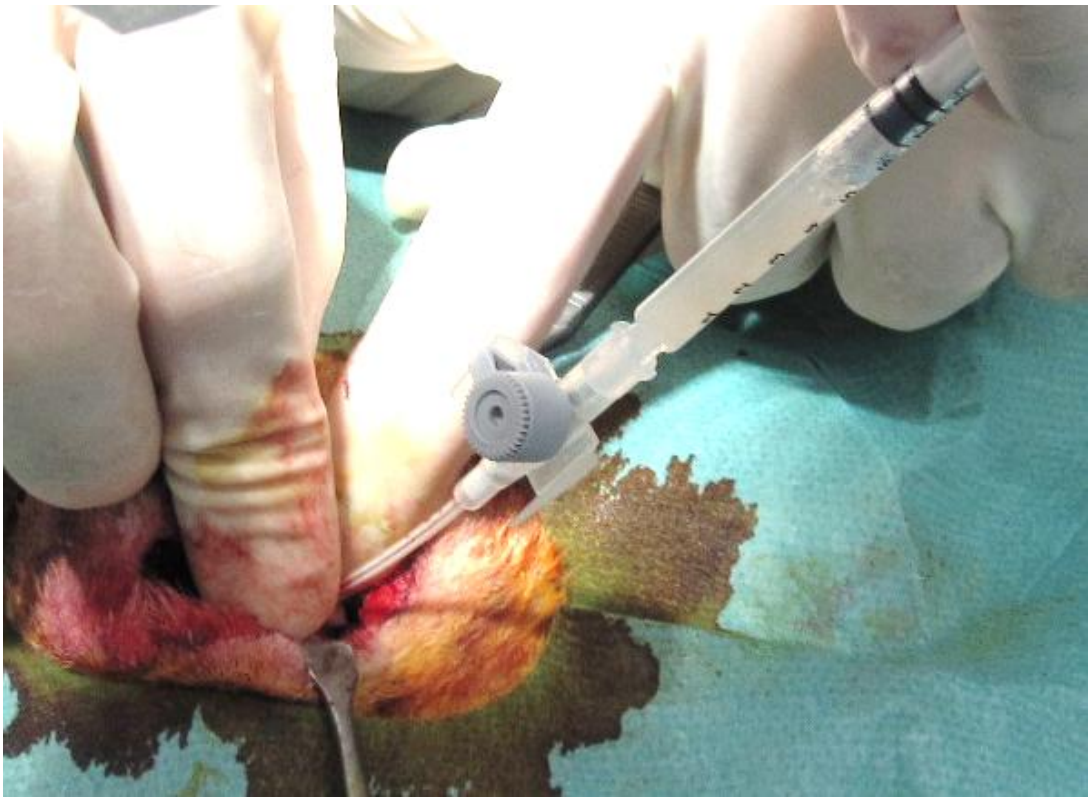

Figure 10 – The injection of the stem cells in the suhchondral region of the femoral head. The hole was sealed using bone wax right after the cannula was drawn back in order to prevent leakage.

Harvesting of femoral heads

The rabbits were sacrificed in CO2 cage. The furs on the both thighs were completely removed. The surgical site was disinfected using baticon. A lateral skin incision was performed over the great trochanter. The incision was extended distally 2 cm over the lateral aspect of femur and 2 cm proximally making a slight curve posteriorly. After the subcutaneous tissue was incised according to the skin incision, tensor fascia lata was split open. Gluteus medius muscle was detached from great trochanter and elevated anteriorly. The anterior aspect of hip capsule was visualized and a "T" cut was performed on the capsule. An osteotomy was carried out at the basocervical region of the femur (Figure 11). The proximal part was detached from all soft tissue connections and was put in saline solution and was sent to laboratory. Layers were sutured appropriately. The same procedure was carried out on the contralateral hip.

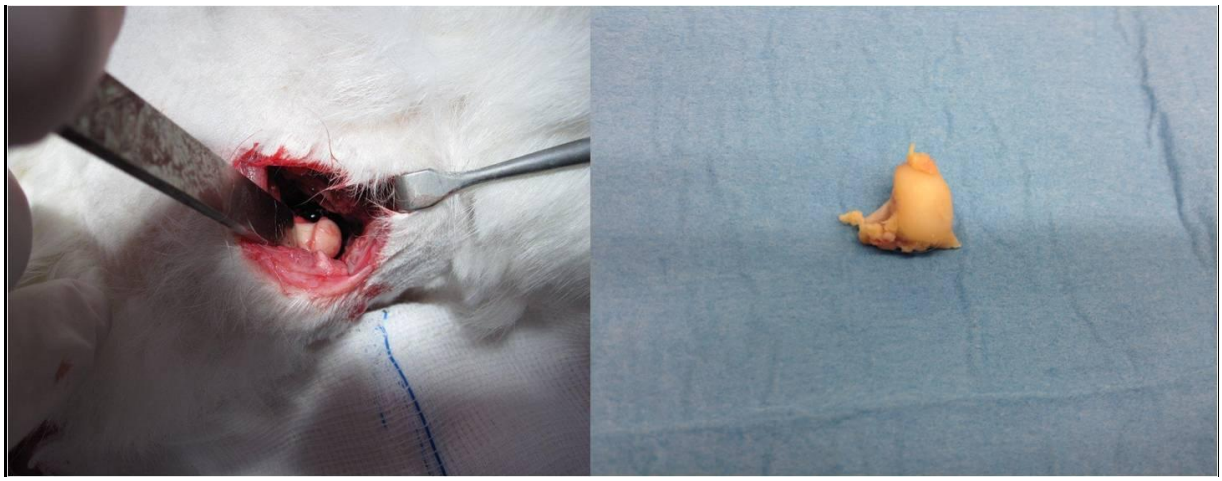

Figure 11 - For histological analysis, the femoral head was excised using an

Histological Assays

Explanted femoral head were fixed with 10% buffered formalin for 2 days, decalcified in fast decal solution composed by 5% acid formalin and 5% of trisodium citrate (BDH Laboratories Pool, England) in dd water for 21 days and then paraffin embedded, as similarly described for animal and human bone specimens. Cut sections (3µm-thick) were deparaffinized and stained with Hematoxylin and Eosin (H&E, Carlo Erba).

Both, control samples and BM-MSC treated samples were examined using a Zeiss Axioscope (Carl Zeiss, Germany) with either 10x or 40x magnifications to detect the elements characterizing the ON event reported, like the presence of empty lacuna or pyknotic nuclei of osteocytes within the bone trabeculae, the cell necrosis of bone marrow, BM fat degeneration, thrombosis and finally the articular cartilage. In parallel, the bone matrices of both groups were evaluated for living osteocytes surrounding the necrotic bone area, index of a repair event. Osteogenesis in control group would mean that animals had an own repair ability to form new bone after a ON, therefore, to assess the efficacy of BM-MSC engraft, we evaluate the amount of new bone tissue in the treated groups versus control group.

Quantification was performed considering replicate specimens (n=3) from each animal in both groups. Photomicrographs were acquired at magnification of 20x with an Axiocam-IcC3 color camera and Axiovision 4.8.2 software (Carl Zeiss). In each photomicrograph the amount of necrotic bone and new formed bone was measured using the Axiovision-4.8.2 software

(Carl Zeiss). In parallel bone lining cells surrounding the new bone formed were scoring in according to the literature.

Statistical Analyses

All data collected was presented as means ± standard error of the mean (SEM). Statistical analysis was performed using the paired Student's test. *P*-values <0.05 were considered statistically significant.

CHAPTER 3

RESULTS

ONFH MODEL VALIDATION AND DECISION OF TIMING

Six rabbits underwent femoral head ON induction procedure. Two rabbits at a time were sacrificed with 2 weeks interval, starting from the end of 2nd week.

Histological features of the femoral heads after ON induction procedure

At the end of 2nd week

Histology of the femoral heads harvested at the end of second week showed typical osteonecrotic changes including fatty degeneration of BM and empty bone lacunas. There were no sign of new bone formation (Figure 12).

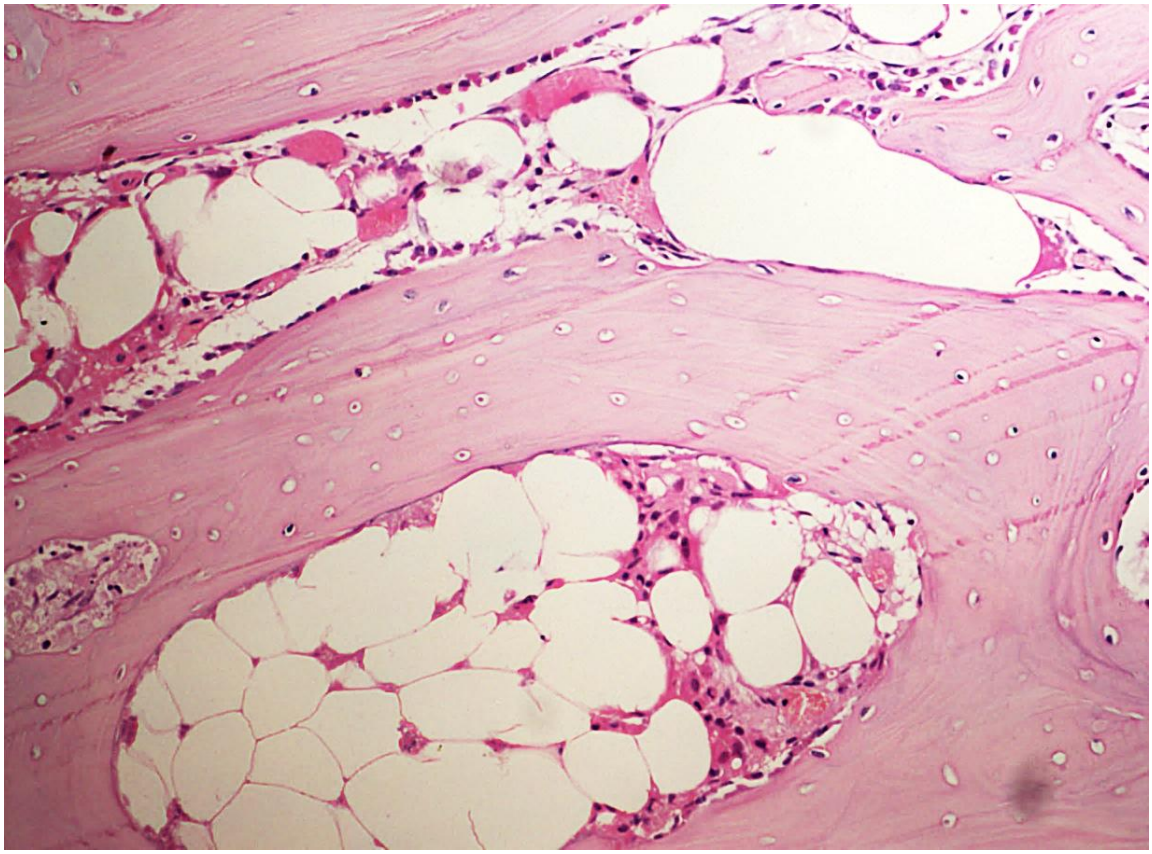

Figure 12 - After damage induction we were able to observe relevant change in rabbit bone and marrow architecture with a robust reduction of marrow hematopoietic cellularity the appearance of stromal fibroblasts, foamy histiocytes and necrotic fatty marrow. Typically bone progressively loose living osteocytes generating a pattern with empty lacunae

At the end of 4th week

Femoral heads harvested four weeks after ON induction showed worsening of ON features including defective bone showing numerous empty lacunae lacking osteocytes and accompanying necrosis of the BM with adipocytes hypertrophy. However, osteoblasts appeared surrounding the necrotic bone depicting the initiation of new bone formation (Figures 13, 14)

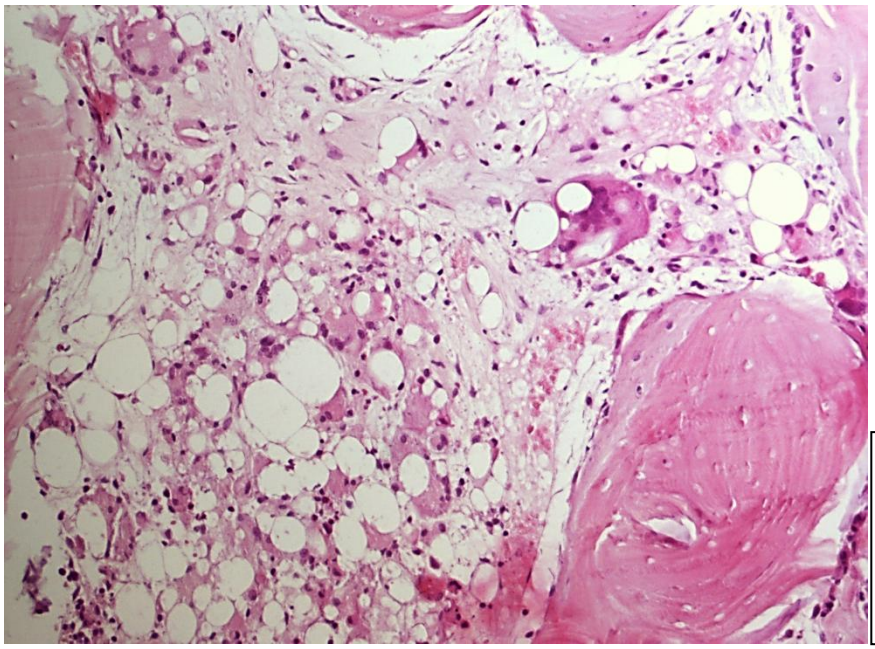

Figure 13 - Four weeks after ON induction, marrow space is filled with adipocytes and fibrotic tissue. ON is well-established

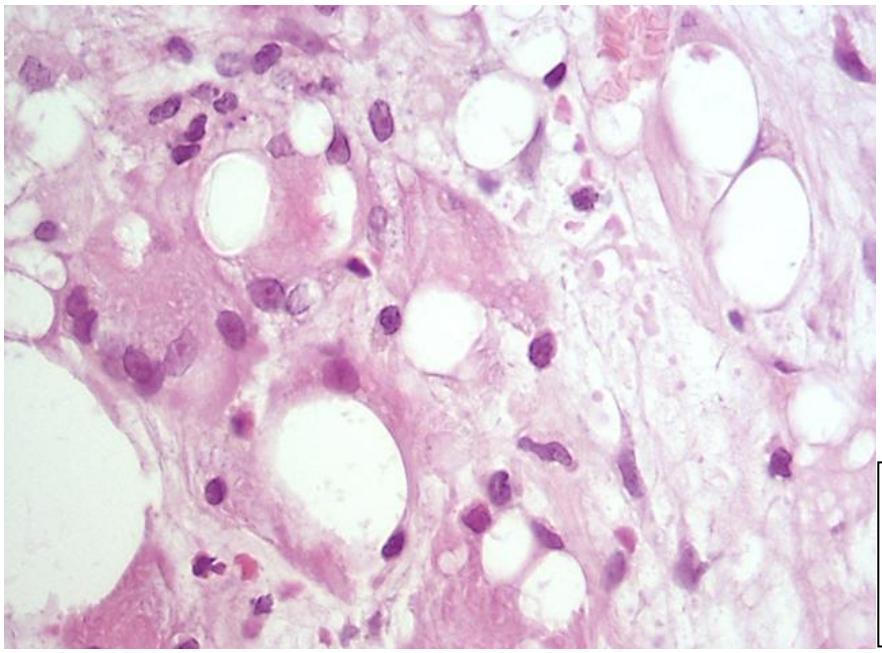

Figure 14 - Osteoblasts appeared surrounding the necrotic tissues which is the sign that auto - repair process started

At the end of 6th week

Femoral heads harvested four weeks after ON induction showed areas of spontaneous bone regeneration. The necrotic bone, with no visible osteocytes, is slowly resorbed and simultaneously replaced with new viable bone. This incorporation process is termed "creeping substitution" as described in humans. Ephiseal areas also revealed large areas of necrotic marrow suggesting a wide damage induction (Figure 15, 16).

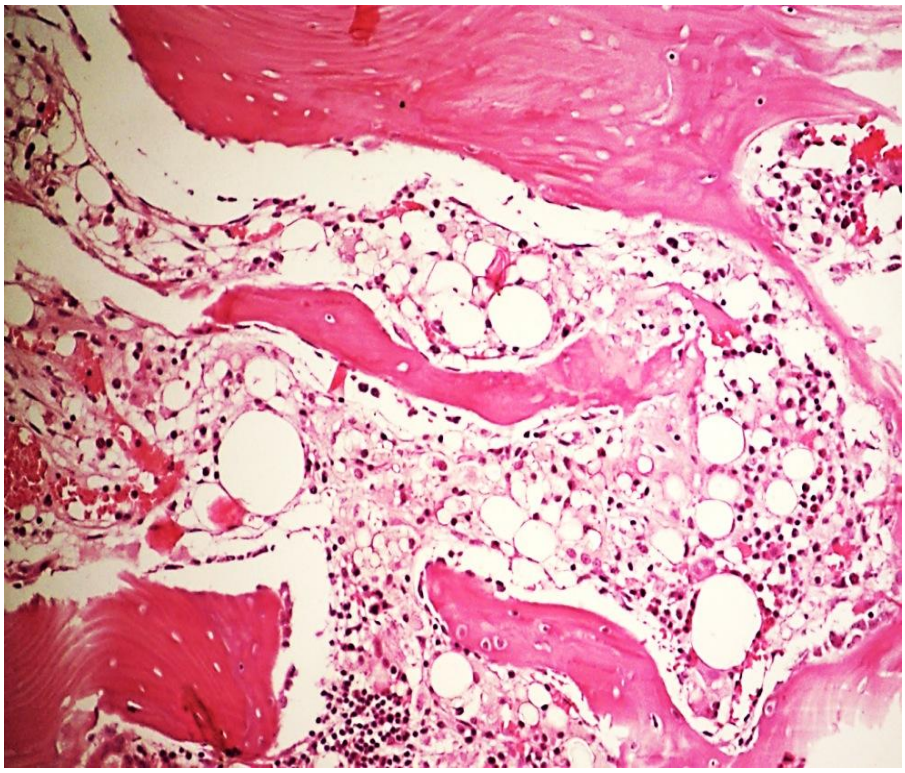

Figure 15 - Medullary cavity is compromised, disorganized and filled with granulation tissue. There are still empty lacunae and fatty degeneration

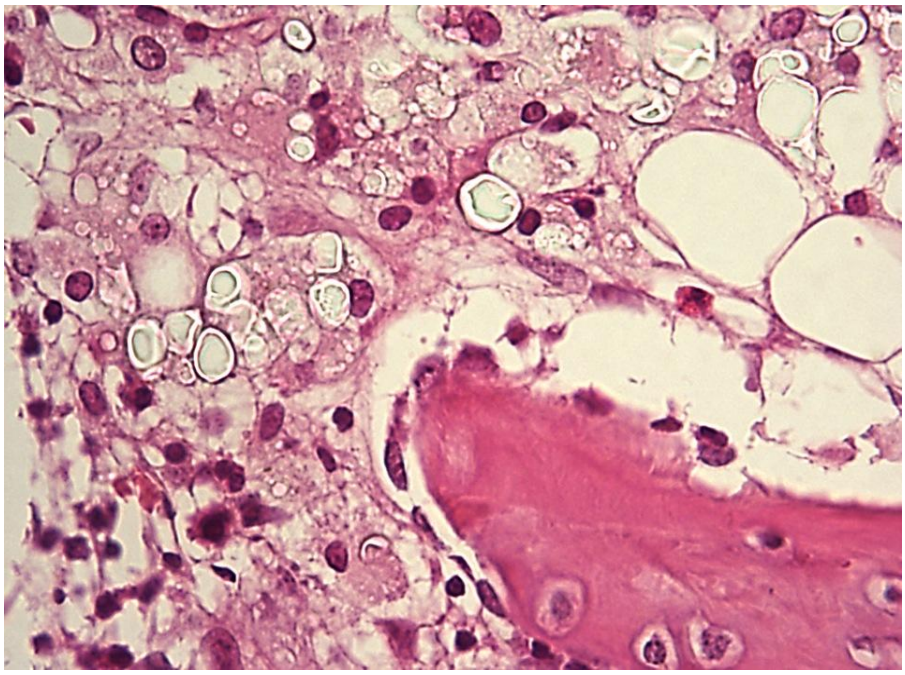

Figure 16 - Osteoblasts started apposition of new bone formation around the necrotic trabeculae (creeping substitution)

Expansion of Stem cells

In vitro experiments indicate that BM-MSC can reach $109x10^6$ in 3 weeks. BM-MSC revealed a typical plastic-adherence and fibroblast shape morphology (Figure 17), lack of CD45 and are positive for CD90 (Figure 18, 19) as recognized marker for rabbit MSC.

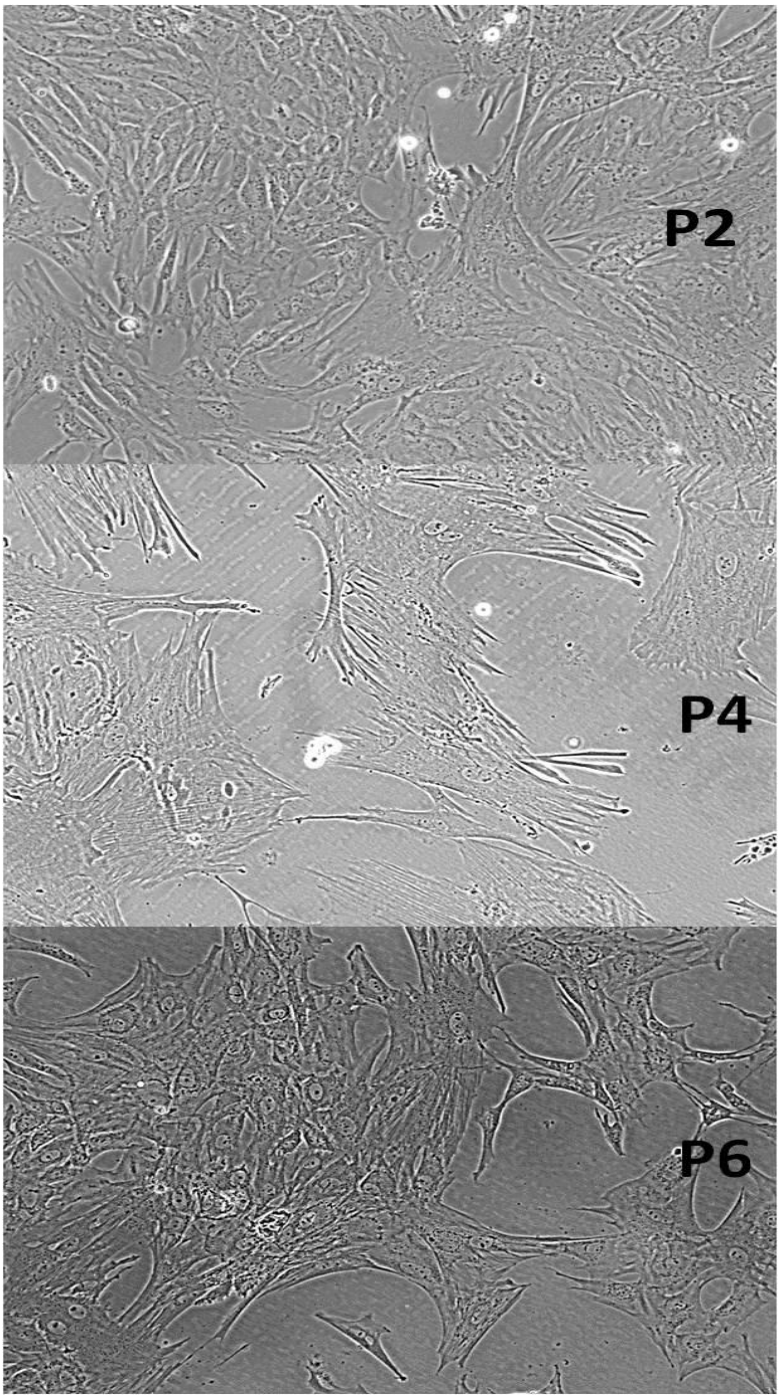

Figure 17 - BM-rMSC in culture in different passages

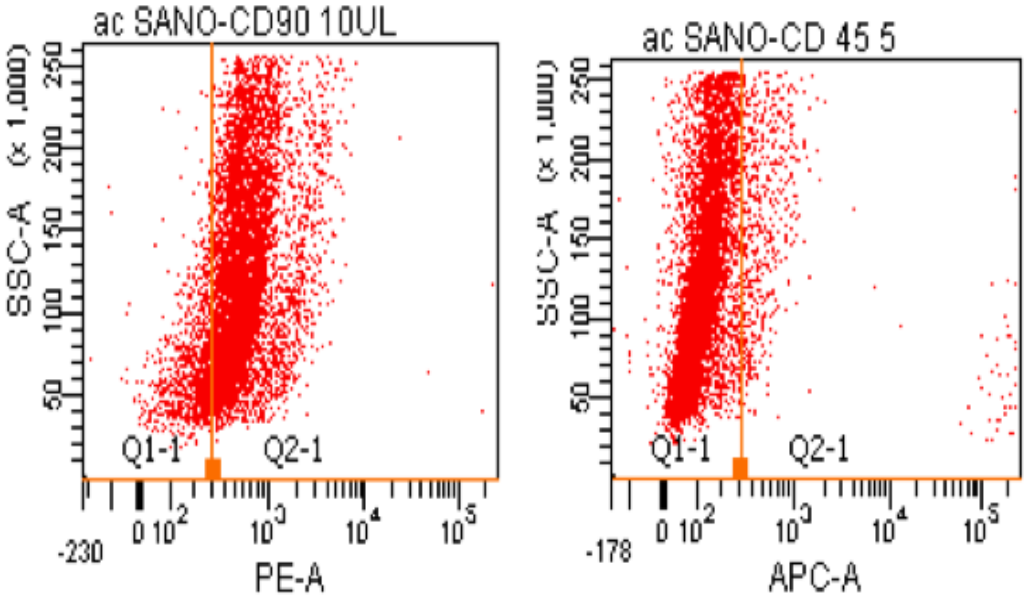

Figure 18 - BM-rMSC FACS analyses on Propidium Iodine negative BM-rMSC

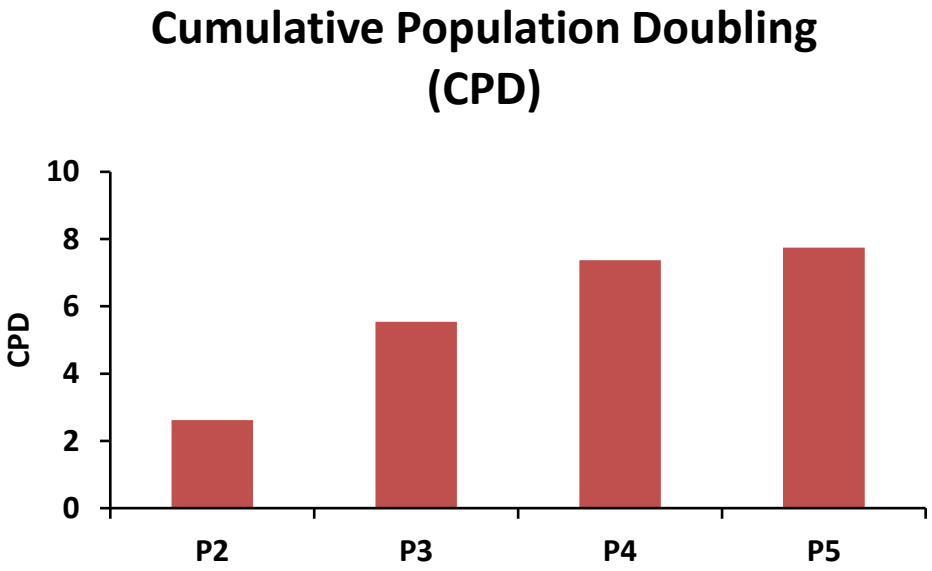

Figure 19 - Four passages were enough to reach the maximum number of stem cell population

Decision of Timing

2 weeks was the limit for the progression of the necrosis and initiation of the repair. At the 4th week, both spontaneous repair and necrosis were visible that would allow to see if the effect of therapy with stem cells would increase the healing. Thus, the procedures were scheduled as follows:

Time 0: harvesting of bone marrow from the right supracondylar region of right femurs of 10 rabbits for isolation and expansion of stem cells

Time + 1 week: ON induction procedure on both femoral heads of 10 rabbits and intramuscular injection of 20 mg/kg methylprednisolone

Time + 3 week: engraftment of 2 000 000 cells in 100ul normal saline on right femoral heads of 10 rabbits (left femoral heads were left as controls)

Time + 5 week: sacrification of the first group of rabbits (5 rabbits) and harvesting of both femoral heads (2 weeks from damage induction)

Time + 7 week: sacrification of the first group of rabbits (5 rabbits) and harvesting of both femoral heads (4 weeks from damage induction)

COMPARISON BETWEEN TREATED AND CONTROL FEMORAL HEAD SAMPLES

To assess the capacity of the transplanted BM-rMSC to generate novel bone, rabbit were sacrificed and transplanted versus non transplanted femoral heads were harvested and processed for histological analyses. In all the specimens, both transplanted and not-transplanted controls (CTL), it was possible to observe different levels of adipocytic hypertrophy in particular at the epiphyseal level. Empty lacunae and oscteocytes with pycnotic nuclei were still present in the different areas (Figure 20).

However, careful analyses of different bone areas indicate that in the non transplanted CTL rabbit the marrow was containing small elongated and irregularly distributed fibroblast stromal elements mixed with disperse hematopoietic cells. On contrary in the BM-rMSC transplanted femurs, the marrow was more densely populated by more cuboidal stromal

elements and fibroblast stromal elements appeared regular and oriented mixed with a more heterogeneous hematopoietic cellular population (Figure 21).

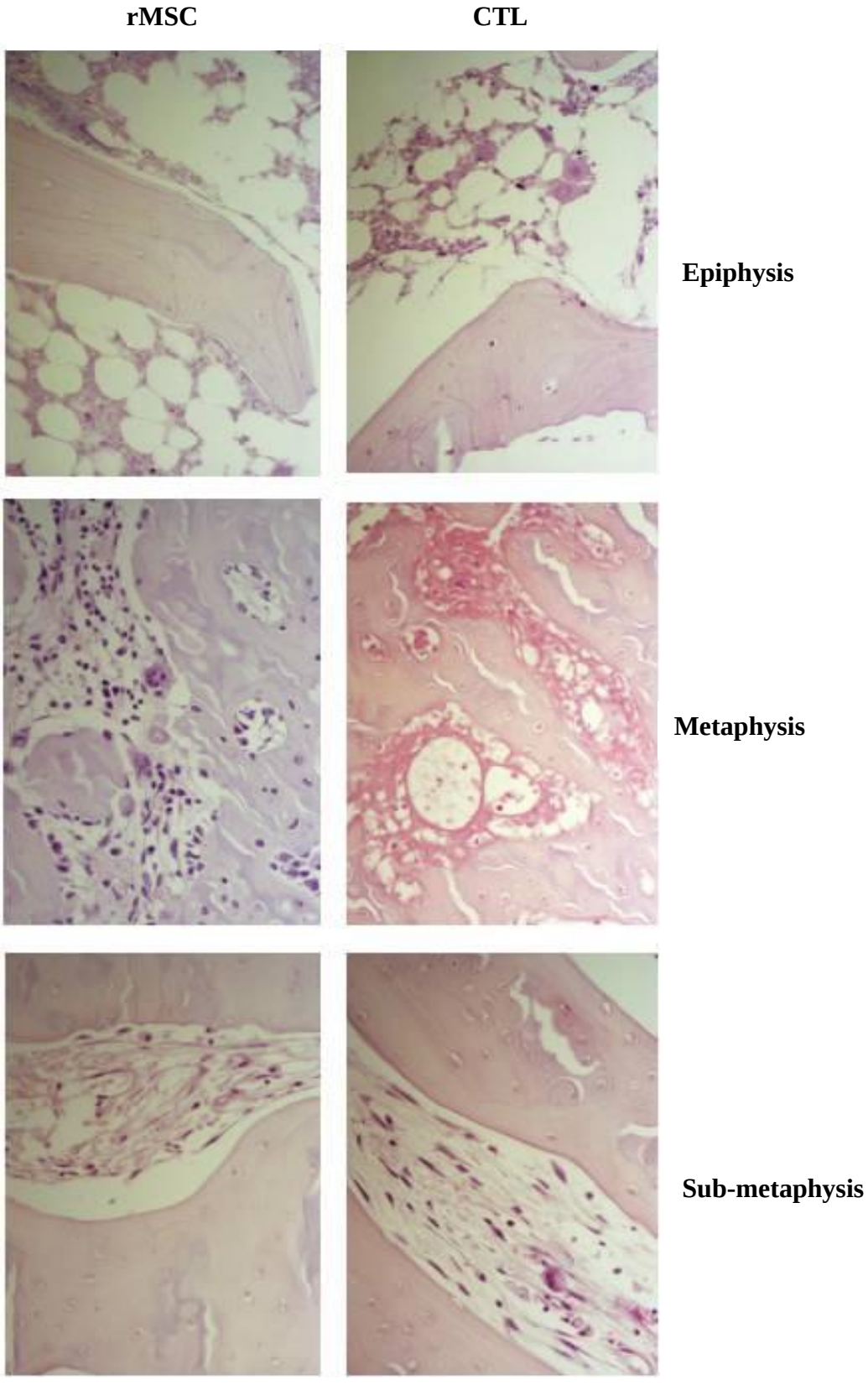

Figure 20 - Osteonecrotic damage in control and MSC transplated

This was particularly evident at the metaphyseal level and in the epiphyses, while no substantial differences were observed at the sub-metaphyseal level and in the proximal diaphysis.

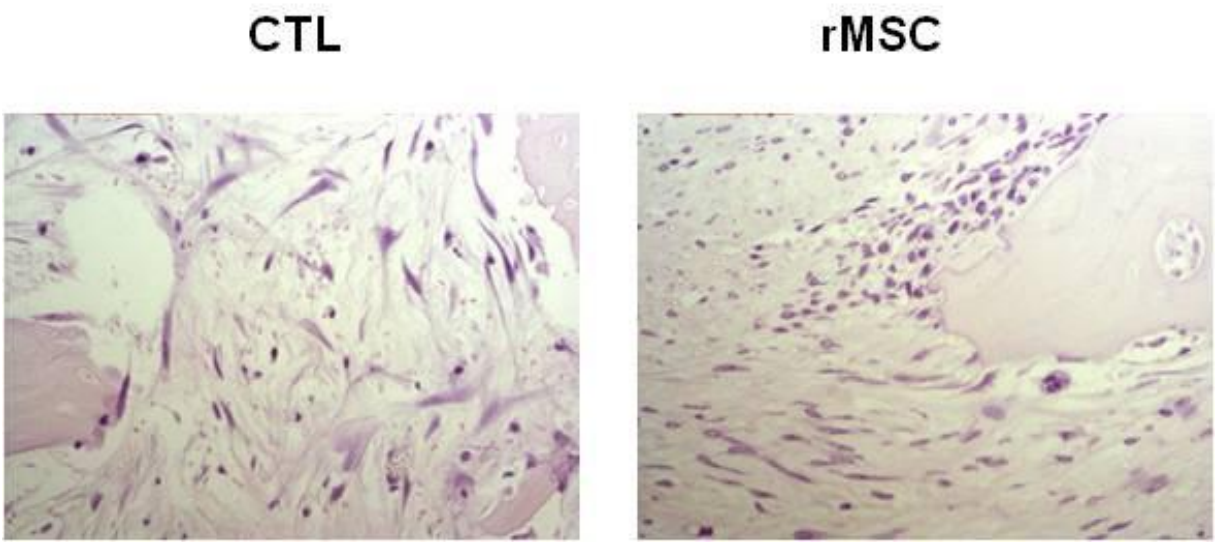

Figure 21 - Fibroblast and cuboidal stromal elements

Further analyses revealed the presence of a reactive osteoblastic rim in the control rabbit specimens which was particularly evident at the metaphyseal level versus the epiphyses. The beginning of creeping bone regeneration on the top of bone devoid of osteocytes was present in the CTL samples as well as in the transplanted at the epiphysis. More interestingly, only the rMSC transplanted femurs revealed the presence of a peculiar ossification mimicking an endochondral ossification with a new bone tissue constituted by matrix and densely populated osteocytes (Figure 22). The newly formed bone tissue was often lined with cells resembling osteoblasts. To quantify these qualitative assessments, a scoring system was then implemented. As seen in Figure 23, the osteocytes density in the rMSC transplanted femurs was significantly higher ($*p<0.05$; T-test) than in the non transplanted control indicating a significant benefit of MSC transplantation in our ONFH model.

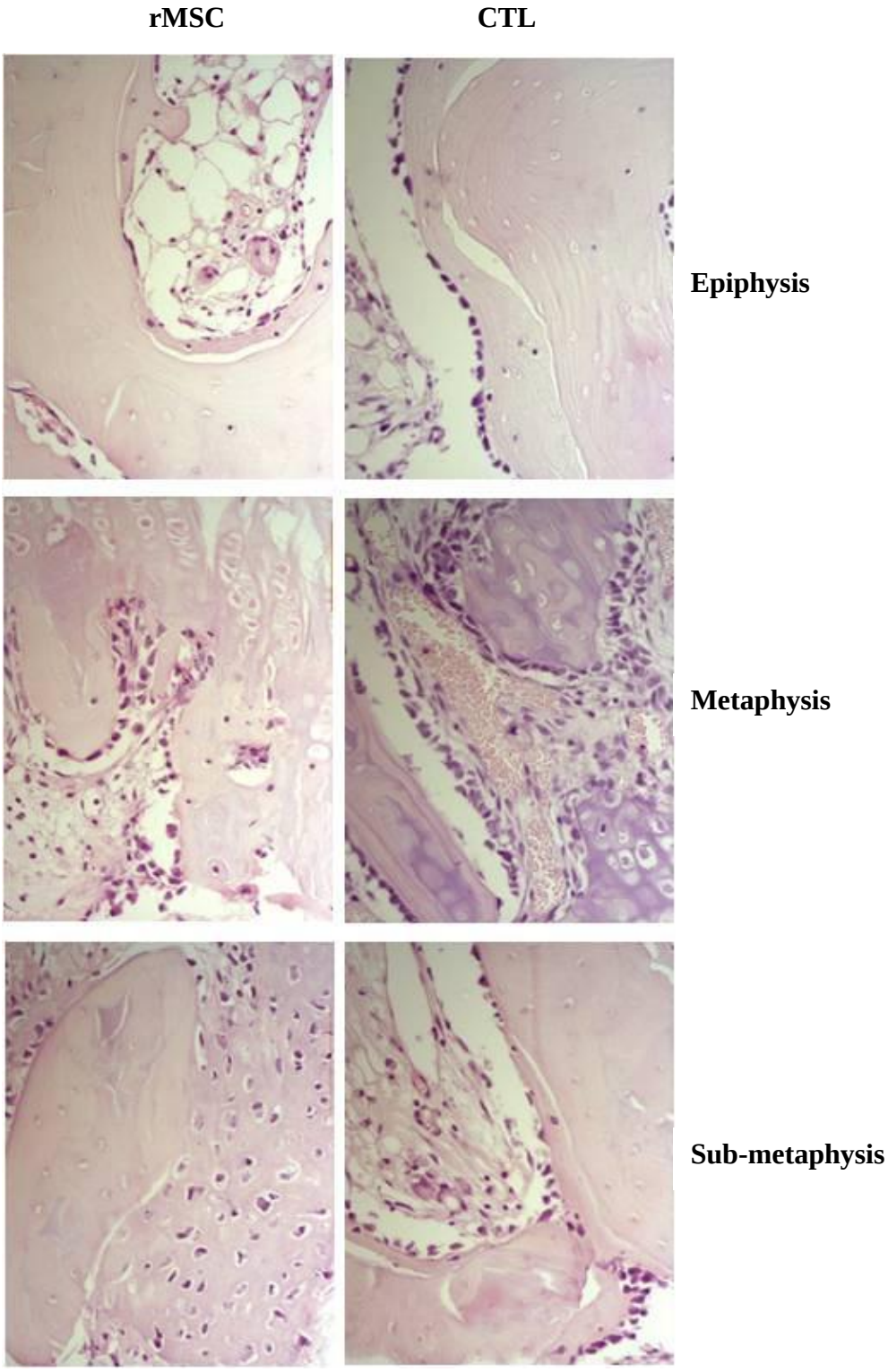

Figure 22. Bone regeneration after ON damage in control and transplanted rabbits

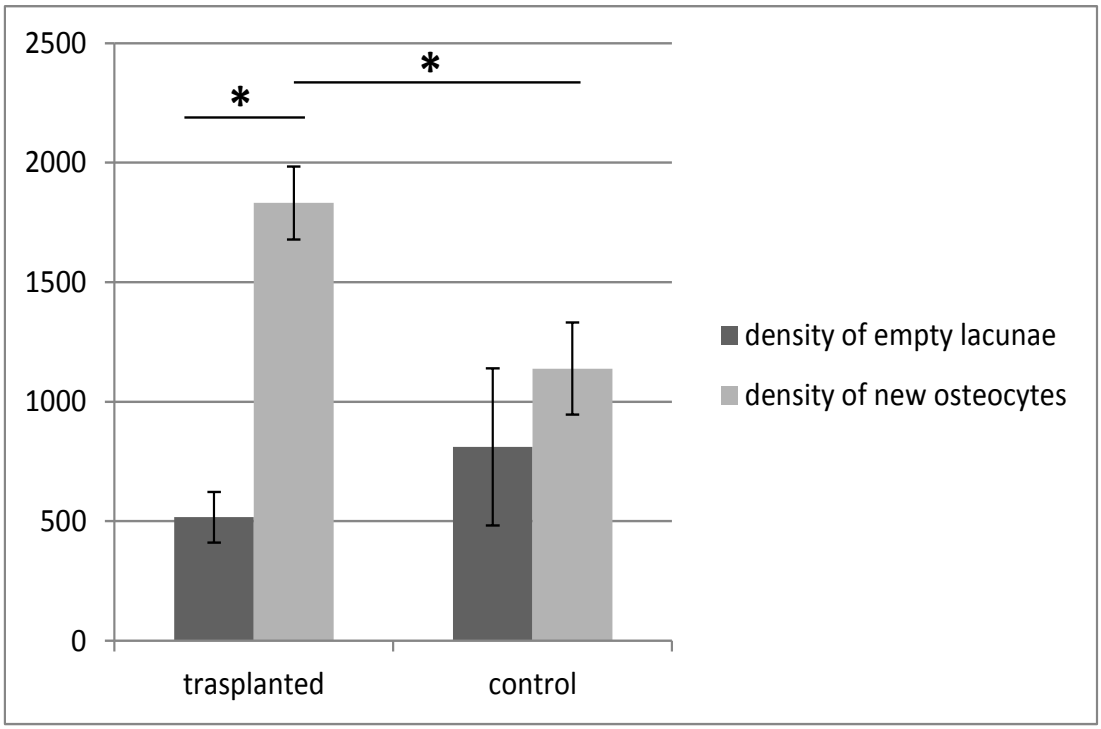

Figure 23 - Assessment of bone density in terms of empty lacunae and new osteocytes in transplanted and control rabbits considering necrotic areas and newly formed bone, respectively. Numbers are related to n=4 20x high-power fields/rabbit for triplicate specimens.

CHAPTER 4

DISCUSSION and CONCLUSION

DISCUSSION

Corticosteroids provide considerable benefit to patients who have received bone marrow transplantation, organ transplantation or suffer autoimmune disease. However, a particularly severe potential side effect is corticosteroid-induced ONFH, with a reported incidence in BMT patients of 19%. Numerous factors have been associated with corticosteroid-induced ON including fat embolism, haemorrhage and increase of intraosseous pressure with the outcome of an interrupted blood circulation to the femoral head that leads to apoptosis of the tissue-supportive cells.

Osteonecrosis of the femoral head is a common disorder in a relatively young patient population, with an average age of mid-thirties [81]. It is estimated to afflict approximately 15,000 new human patients per year in the United States [112]. Traumatic and non-traumatic ON of the femoral head is assumed to have an ischaemic origin and blood supply disorder although the pathomechanism of ON is still not clear. However, it is impressed that the pathology is quiet similar even with variable etiological factors [7]. ONFH progresses and leads to joint pain, loss of function, collapse of the femoral head and hip joint destruction. To date, many studies have reported on prevention and treatment of ONFH using animal models. Although differences of ON development mechanism exist between humans and animals, there may be several common pathways which lead to ON development [87]. Successful prevention and treatment of ONFH is hampered by the lack of an ideal experimental animal model of the disease.

Several rabbit models have aimed to recapitulate the pathology. The comparative bone microanatomy of the femoral head across species shows considerable variation, justifying our approach of addressing multiple animal models, yet there is similar mineral density for the cancellous bone at the femoral head in most species. In our study, to ensure more consistent osteonecrotic lesions in the rabbit, electrocoagulation of the soft tissue attachments at the femoral surgical neck was performed circumferentially to interrupt the extraosseous blood supply to the femoral head through the medial and lateral circumflex arteries. In addition, intramuscular injection of methylprednisolone was performed to interrupt the endosteal circulation. With this combined method, we were able to observe relevant change in bone and marrow architecture. Focusing on bone histology we observed features typical of the human ONFH, including defective bone showing numerous empty lacunae lacking osteocytes and

accompanying necrosis of the bone marrow with adipocytes hypertrophy. These features progressively worsened until 6 weeks from damage induction. However, spontaneous bone regeneration was visible starting from the 4th week. This creeping substitution capability of rabbits did not allow us to study the advanced stages of the disease and obliged us to perform the stem cell therapy as described in humans. Epiphyseal areas also revealed large areas of necrotic marrow suggesting a wide damage induction. Therefore, taking into account the histological findings and the time of damage induction we confirmed that combining surgical procedures with blood supply occlusion and steroid delivery it is possible to obtain a ONFH model in rabbit.

Since the relationship between the occurrence of ON and the systemic administration of corticosteroids was first reported in 1957, there have been several experiments that demonstrated many steroid-related histopathologic and pathophysiologic alterations in the bone tissue, such as fat embolism, an increased pressure of bone marrow in the femoral head, an enlargement of the fat cell size, and an accumulation of lipid within the osteocytes [56, 65]. As a result of these studies, intramuscular injection of high dose corticosteroids is widely used to induce ON various animal models [37, 88, 115, 122, 129, 188, 196]. Commonly used corticosteroids are prednisolone sodium succinate, methylprednisolone acetate and triamcinolone acetonide. A single high dose intramuscular injection of methylprednisolone is shown to induce ON successfully. However, sole corticosteroid injection is not able to induce ON in the epiphyseal region. ON-prone sites may differ in mammalian species, and thus the distribution of ON should be characterized by examining all parts of the bone. Most previous studies have mainly examined the femoral head, expecting a collapsed lesion [65, 88, 189]. The differences observed among species may result in the difference in the location of ON. Moreover, in humans, ON is known to occur in multifocal regions in various part of bone tissue, and even the ON occurring in the femoral head sometimes remains silent and does not collapse if the region is small or is located in the central portion [112, 137]. Whether the ON region undergoes collapse or not is mainly due to the size and location of the ON against the weight-bearing area, and we should also keep in mind that ON undergoing collapse is only one part of all the ON that occurs in humans.

Matsui et al induced vasculitis in animals by injecting horse serum and then a large amount of hormone, which produced typical ON [118]. Small arteries are the target organ of vasculitis, which is caused by the binding of immune complex to the blood vessel wall. Subsequent administration of hormone inhibits the synthesis of collagen and elastic protein, aggravating

the contraction of the blood vessels with vasculitis, platelet aggregation, and necrosis of endothelial cells, and causing breakdown and obstruction of small arteries [158]. The processes led eventually to osteonecrosis in the bone. However, Matsui et a1 reported that ON was recognized in only the metaphysis and diaphysis in rabbit models of ON produced by serum sickness with horse serum and corticosteroid treatment [118]. Wen et al, combined serum sickness model with prednisolon injection and showed osteonecrotic changes in the femoral head [192]. However, this method had increased animal mortality rates and was not proper for our study.

Qin et al, used a combination of lipopolysaccharide and methylprednisolone to induce ON [148]. Histopathologically, they were able to identify ON lesions in multi-focal regions, including locations at both proximal and distal femur. In addition, reparative appositional bone formation was also presented around the necrotic bone and reparative fibrous granulation tissues were found next to the necrotic bone resorption. In their study no rabbit died as a complication of the procedure which demonstrated that this technique was fairly safe. However, since the induction of ON in the epiphyseal region was as low as 29% we did not choose this method as ours.

Tissue destructive agents have been used to create ON [172]. Zhang-hua et al applied liquid nitrogen directly to the cartilage overlying the femoral head of rabbits. They demonstrated that, two weeks after surgery, the histological examination showed necrosis of cartilage cells, osteocytes and bone marrow fat cells around the frozen area. Four weeks after surgery, hematopoietic tissues in the bone marrow was significantly decreased and the cells and reticular formation were loose. The surface of femoral head was pronouncedly coarse and articular cartilages were denudated in some area. The trabecular bone was disturbed and a fraction of trabecular bones were thinned or had been creased. Six weeks after surgery, the amount of hematopoietic tissue further decreased and the injury of articular cartilages was deteriorated. The area of denudated cartilages enlarged and subchondral bone was exposed. Bones were damaged and trabecular bones were irregularly arranged or absorbed or had been creased. The lacunae were vacant and the amount of newly generated. These findings showed that this method would be ideal to study the advanced stage of the disease. Manggold et al, applied purified ethanol directly into the femoral head of the sheeps through an opening just below great trochanter [111]. They documented partial necrosis in all animals over a period of 12 weeks. Li et al., applied heat to the femoral head by using a microwave antenna [104]. They reported femoral head collapse in 69% of the rabbits at the end of 3 months. However,

in our opinion these techniques did not reflect the pathogenesis occurring in humans which is due to vascular deprivation rather than direct injury to osteocytes or chondrocytes. In these studies the cartilage cells became necrotic in 2 weeks. In contrast, in real ONFH, the loss cartilage cells occur due to the loss of bony support which happens in advanced disease. Thus we did not prefer to apply any of these methods in our project.

Hwang et al, induced ON in the femoral head by combining single high dose intramuscular injection of methylprednisolone and surgical ligation of extraperiosteal femoral neck vessels by cerclage wire [81]. Their histological findings at the end of 2 weeks showed that this technique was able to induce ONFH. In our opinion this procedure mimicked the pathogenesis in humans since the on developed due to the interruption by blood supply to the femoral head. We did not use cerclage wire. Because lateral approach to the proximal femur and hip dislocation allowed us enough exposure to cauterize the femoral neck circumferentially.

MSCs are precursors of osteoprogenitor cells [156]. They play a key role in cell therapy for bone repair, as they are the best characterized multipotent cells and can now be produced reliably for clinical purposes. According to the diamond concept, MSCs play a crucial role in bone repair [64]. Cell therapy can serve as an alternative to autologous bone grafting. A large number of osteoprogenitor cells are implanted at the injury site, either alone or combined with a matrix. BM MSCs are currently the most appropriate cells for inducing bone repair, as they have a strong osteogenic potential and are easily obtained by culturing iliac-crest aspirates. In our project we were able to demonstrate that application of the MSC and histological showed a significant bone apposition in the transplanted animals.

Encouraging results concerning the cellular origin of ONFH have triggered great interest into further research on the cytotherapy of disease. If non traumatic ONFH is highly related to the alterations of MSCs or other progenitor cells osteogenic differentiation of progenitor cells could be induced in the femoral head, and stem cells therapy could prove to be effective in animal models with ONFH. Asada et al. have evaluated injection of bone marrow cells (BMCs) for the prevention of corticosteroid induced ONFH [9]. The authors concluded that direct injection of autologous BMCs into femurs could prevent corticosteroid-induced ONFH following treatment with high-dose, short-term steroids. Yan et al. employed MSCs to treat ONFH and investigated the survival and differentiation status [198]. Their research demonstrated that the transplanted MSCs could survive, proliferate and differentiate into

osteoblasts directly, which could accelerate the repair process. Although the animal model was induced traumatically in the experiment, the fate of MSCs transplanted to the femoral head should be similar to that in non traumatic ONFH.

Zhang-hua et al, in their study, in which ONFH in rabbits was induced by application of liquid nitrogen, showed that intra-venously implanted MSCs could migrate into the femoral head of hosts, and especially migrate directionally and survive in the necrotic femoral heads [105]. They concluded that, it is feasible and safe to treat femoral head necrosis by intravenous transplantation of allogeneic MSCs.

Hernigou et al. have published clinical data on their experience. They treated 189 hips in 116 patients with autologous BMCs and had a follow-up of five to ten years. Satisfactory results could be achieved in the majority according to the improvement of Harris hip score, radiographic assessment and refusal of total hip arthroplasty. The prognosis was not only highly related to the stage of disease, but also to the progenitor cells transplanted. When patients were operated on before collapse and received a greater number of BMC injections, a better outcome could be expected [77]. In 2008, Hernigou et al. retrospectively analysed 534 hips in 342 patients with ONFH treated with autologous BMC transplantation [78]. The results were really encouraging. They showed that the volume of necrosis would decrease from 26 cm3 to 12 cm3 in 371 patients with an average follow-up of 12 years. There were only 94 patients who progressed to total hip arthroplasty. The author concluded that the best indication for cytotherapy of ONFH was in the pre-collapse stage when the hip was symptomatic. In another report from Gangji et al., two patients with ONFH were treated by injection of BM-MSCs [61]. Osteoprogenitors and osteoblasts from bone marrow were separated and expanded in vitro, and injected into the necrotic zone after differentiation under autologous conditions. Pain reduction, necrotic lesion decrease and functional improvement were recorded in the early period, and only minor side-effects were found. Although these preliminary reports are poorly controlled and need further confirmation, the early signs are encouraging. Combined with previous animal-model research, it seems that the treatment by cell transplantation and replacement could improve the armamentarium for ONFH.

CONCLUSIONS

We were able to recapitulate the protocol for induction of ONFH by treatment with corticosteroids and a surgical procedure in New Zealand White Rabbit according to previously published protocols. Experiments have established the timing needed for autologous bone marrow cell harvest, induction of the ON damage, implantation into the site of the damage and timing needed to explore healing from experimental intervention using autologous MSC versus the spontaneous regenerative capacities of the rabbit. In particular, relevant results have been obtained focusing on bone histology after damage that revealed that we were able to confirm previous observations regarding histological features typical of ONFH, including defective bone showing numerous empty lacunae lacking osteocytes and accompanying necrosis of the bone marrow with adipocytic hypertrophy. Combining traumatic surgical procedures involving ligature to occlude the blood supply or repeated application of the steroid produced a more pronounced ON histopathology within 4 weeks than single-dose application of steroid alone providing a model that was effective at mimicking early stage ON.

Having established appropriate pathology, we next applied autologous BM-MSC intervention to two groups of rabbits, applying the cells in a small volume of saline into the femoral head either 2 weeks or 4 weeks after induction of ONFH by the combination of surgical vascular occlusion and steroid treatment.

The rabbits were sacrificed two weeks after application of the MSC and histological showed a significant bone apposition in the transplanted animals.

Our results reveal that stem cell therapy could modify the unfavorable prognosis of ONFH. However, both preclinical and clinical studies are necessary to confirm that the increased bone apposition in BM-MSC treated animals are due to the stem cell therapy rather than autoregenerative capabilities of individual animals and to show the efficacy of stem cell therapy to prevent the progression of disease in humans where the femoral heads are exposed to weight.

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