Universitat Autònoma de Barcelona

OSTEONECROSIS OF THE FEMORAL HEAD TREATMENT WITH ADVANCED CELL THERAPY AND BIOMATERIALS IN AN EXPERIMENTAL SHEEP ANIMAL MODEL

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Brothers Manuel y Andres

Sister Mariel

My reason and life engine

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INTRODUCTION

1. – Osteonecrosis of the femoral head (ONFH)

1.1. - Definitions and concepts

Osteonecrosis of the femoral head (ONFH) is an acquired disease represented by an ischemic process which progresses to joint deformity and incongruity. In its more advanced state it derives in degenerative joint changes, usually characterized by pain and functional incapacity, which causes an important socioeconomic impact (1). At present, there are no effective treatments to stop progression at its beginning, and the late phases can only be treated with joint reconstruction surgery (2).

Initial treatments include pharmacologic agents, biophysical treatments and/or joint-preserving surgeries (3–5). The most widely used techniques at the early stages of disease development are core decompression (with or without the contribution of non-vascularized graft), rotational osteotomies and vascularized structural bone grafts, although these do not seem to have much incidence except in small to moderate lesions. However, new treatments are in progress, such as combined use of morphogenetic protein or mesenchymal cells, which has yielded good results, at least, for delaying radiologically-evident progression (6–8).

Many different etiological approaches to define osteonecrosis have been presented by a myriad of authors. Choi *et al.* define early stages of necrosis of the femoral head as a bone disease that still does not present subchondral fracture and the adjacent acetabular cartilage is normal. It is diagnosable by magnetic resonance (MRI). In the following stages, it is very common for subchondral fracture to occur,

which can lead to instability and collapse of the femoral head, as well as displacements of the articular cartilage, which progresses towards secondary arthritis of the terminal joint (9).

In a similar way, Castiglioni *et al* define osteonecrosis as a clinical entity characterized by necrosis of the trabecular bone and bone marrow produced by a disruption in the blood flow. It is also known as avascular bone necrosis (NOA), aseptic necrosis and avascular subchondral necrosis (10).

1.2. - Prevalence

Osteonecrosis is a relatively common ailment, accounting for 10% or more of around half a million joint surgeries practiced in the United States per year (1). The age of 75% of the patients with osteonecrosis ranges between 30 and 50 years. Its prevalence is twice in men than in women with a male to female ratio of about 7:3. (11). Patients with systemic lupus erythematosus (SLE) are an exception to this gender ratio (12)

1.3. - Etiology

Wide arrays of risk factors for the development of osteonecrosis of the femoral head have been recognized. Such risk factors include corticosteroid use, alcohol, trauma, hypercoagulable states. However, much of the etiology of the disease is somewhat unclear.

In general terms, the causes of osteonecrosis encompass the interruption of blood supply to the bone and consequent ischemia, due to local trauma or non-traumatic systemic conditions.

Table 1. Common causes of ONFH.

Direct causes	Indirect causes	Rare causes
Trauma	Corticosteroids	Polyarteritis
Irradiation	Alcohol abuse	Thalassemia
Hematologic Disorders	Idiopathic	Carbon tetrachloride poisoning
Cytotoxins	Thrombophilia	Hyperlipidemia
Gaucher disease	Hypofibrinolysis	Cushing's disease
Sickle cell disease	SLE, other autoimmune disorders	Pregnancy
	Renal insufficiency	
	Organ Transplantation	
	Hemophilia	

Among the traumatic causes of osteonecrosis are: burns, fractures, dislocations, vascular trauma and Kienböck's disease. The non-traumatic causes of osteonecrosis comprise a wide range of pathological situations, which have been classified as follows (13).

~ *Hematologic*

- Hemoglobinopathies: sickle cell anemia, thalassemias.
- Disseminated Intravascular Coagulation (DIC)
- Polycythemia
- Hemophilia

~ *Metabolic / endocrinological*

- Hypercholesterolemia
- Gout
- Hyperparathyroidism
- Hyperlipidemia
- Pregnancy
- Cushing disease
- Chronic renal failure
- Gaucher disease
- Diabetes (obesity)
- Fabry disease

~ *Gastrointestinal*

- Pancreatitis
- Inflammatory bowel disease

~ *Neoplastic*

- Marrow infiltrative disorders

~ *Infectious*

- Osteomyelitis
- Human Immunodeficiency Virus (HIV)
- Meningococcemia

~ *Vascular / Rheumatologic / Connective tissue disorders*

- Systemic Lupus Erythematosus (SLE)
- Polymyositis
- Polymyalgia Rheumatica
- Raynaud Disease
- Rheumatoid Arthritis
- Ankylosing Spondylitis
- Sjögren's Syndrome
- Giant Cell Arteritis
- Thrombophlebitis
- Lipid Embolism
- Ehler-Danlos Syndrome

~ *Orthopedic problems*

- Slipped Capital Femoral Epiphysis
- Congenital Hip Dislocation
- Hereditary Dysostosis
- Legg-Calvé-Perthes Disease

~ *Extrinsic dietary / environmental factors*

- Dysbaric conditions (Caisson Disease)
- Alcohol consumption
- Cigarette smoking

~ *Iatrogenic*

- Corticosteroids
- Radiation Exposure
- Hemodialysis
- Organ Transplantation
- Laser Surgery and

~ *Idiopathic causes*

It is widely recognized that the lack of blood flow to the bone is the common denominator for the development of osteonecrosis. If the cause is traumatic in nature, then the physical interruption of regular blood supply is the event leading to necrosis. Whereas if the etiology is non-traumatic, the pathogenesis is not always as straightforward. The process will depend on the underlying condition and may or may not comprise coexisting morbidities. While many risk factors for developing osteonecrosis have now been acknowledged, they do not necessarily predict the disease or its outcome. ONFH is rather, a multifactorial on-going condition (14) as we will further discuss in the pathogenesis-related section.

2. – Diagnosis of ONFH

Currently, there is a better understanding of the natural history of symptomatic osteonecrosis and how it progresses, than the myriad of the commonly associated factors responsible for its development.

Surprisingly, many patients with osteonecrosis often develop the disease within few months of exposure to a particular risk factor, whereas other patients never develop osteonecrosis when exposed to that same risk factor. The more knowledge we are able to acquire regarding the natural history of ONFH would allow us to better understand disease prognosis and outcomes so that management results in the best line of therapy for a given patient

2.1. - Symptoms associated with ONFH

Osteonecrosis of the femoral head may lead many different courses mainly depending on the site and size of the ischemic structure. The presenting symptom is usually pain, which may be initially mild or as happens with more insidious onsets, the pain may be vague, making the diagnosis less evident. On the other hand, when the cause is traumatic, the pain may be of rapid onset and severe (15). In cases where ischemia is extensive, infarction of the bone causes very intense pain and is usually the result of predisposing conditions such as sickle cell hemoglobinopathies, lipid storage diseases, etc. (16).

Initially, non-traumatic osteonecrosis may be asymptomatic, and the duration of this stage may be variable (17). It seems that in many cases the pathophysiology of pain is related to the increase in pressure inside of the bone structure. Although

pain may also be the result of synovial effusions, degenerative arthritis, etc., it is crucial to determine the etiology of the pain in order to successfully resolve the cause with precise treatment.

In osteonecrosis of the femoral head, pain usually involves the groin or anterior thigh area. Frequently, the disease is unilateral but may become bilateral in 55% of the patients over the course of 2 year (17). Pain may further be induced by joint mobilization, until it becomes present even at rest for which analgesics are commonly prescribed. Mechanical function of the joint may be initially preserved but as the disease progresses, motion may become limited and painful. Many times, limping may be present in otherwise radiographically normal subjects.

Some studies have reported that symptomatic osteonecrosis lesions appearing at the weight-bearing regions of the femoral head show high rates of collapse (18,19). Additionally, development of subchondral fractures usually predicts posterior collapse. Collapse of the articular surface is an irreversible event occurring in the natural history of osteonecrosis. This collapse commonly presents with concomitant pain, installment of osteoarthritis, and a poor prognosis.

2.2. - Imaging diagnosis of ONFH

Imaging diagnosis of osteonecrosis may include different methods such as plain radiography, magnetic resonance imaging (MRI), computed tomography (CT), radionuclide examination, and PET-CT (20,21).

The role of imaging has multiple clinical goals such as establishing differential diagnoses of ONFH that also exhibit hip pain such as confirming clinically suspected ONFH in high-risk patients, screening skeletal structures for lesions, staging of disease for optimal treatment planning, monitoring treatment and explain possible complications of the disease or its treatment.

2.2.1. - Plain radiography

The low-cost and easily available plain radiograph is usually the first tool on the diagnostic process of ONFH; its characteristic features and the progression of the disease are easily visualized by this method. Despite its limitations in detecting early, preradiographic stages, plain radiography has been widely used for the classification of osteonecrosis. Radiographic findings of ONFH include:

(a) Sclerosis surrounding an osteopenic area. A sclerotic rim might imply reactive bone remodeling at the necrotic viable osseous junction. This pattern characterizes the stage II according to the modified Ficat–Arlet, Steinberg's and ARCO systems.

(b) A crescent lucent subchondral line resulting from a subchondral fracture. The presence of the "crescent" sign in the absence of segmental flattening, classifies the lesion as stage III in all major staging systems.

(c) Segmental flattening of the femoral head with or without joint space narrowing and secondary osteoarthritis. This pattern is consistent with advanced ONFH (figure 1).

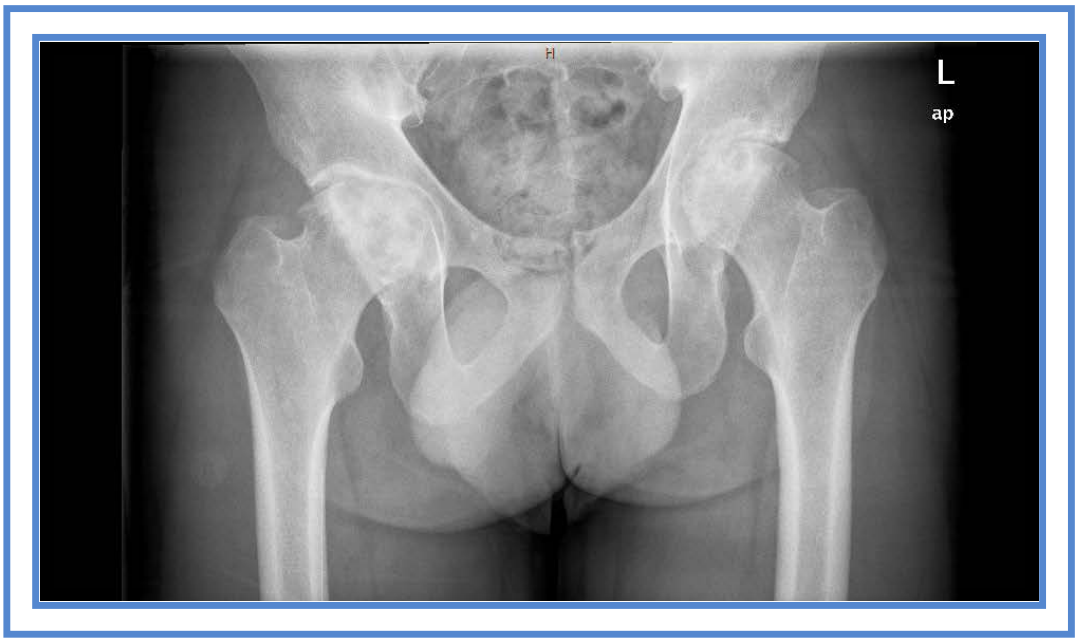


Figure 1: Bilateral ONFH with advanced radiological signs of osteoarthritis.

The detection of the disease by radiographies started as early as the 1960s by Ficat and Arlet and revised in the 1970s. From these researchers, several classification systems were proposed, including Steinberg (22), which follows the progression and extension of osteonecrotic lesions fairly accurately and in six stages:

Stage 1. Normal X-rays, abnormal MRI or bone scan

A <15%

B 15-30%

C> 30%

Stage 2. Abnormal lucency or sclerotic site in the femoral head

A <15%

B 15-30%

C> 30%

Stage 3. Subchondral collapse without flattening of the femoral head

A <15%

B 15-30%

C> 30%

Stage 4. Flattening of the femoral head, normal space of the joint.

A <15%

B 15-30%

C> 30%

Stage 5. Narrowing of the joint space, acetabular changes, or both

Stage 6. Advanced degenerative changes

During the early stages, signs of sclerosis surrounding osteopenic areas might be evident but understated. As the disease develops it is characteristic to find sclerosis, cystic changes and the pathognomonic subchondral crescent-shaped lucent lesion. In the later stages of disease progression, the femoral head flattens and loses its sphere-shaped structure due to subchondral collapse. Finally, an arthritic degeneration follows which might include changes on the acetabular side.

Plain radiography is an ideal tool for the initial management of hip pain as it is low-cost, simple and readily available. The presence of a subchondral fracture is evident radiographically when the “crescent sign” is existent, which demonstrates advanced stages of osteonecrosis that might lead degenerative joint disease.

Although still considered the basic initial imaging for suspected ONFH, radiographs exhibit high specificity for advanced disease but low sensitivity for early disease. Portrayal of early disease is crucial given that early diagnosis is directly related with better prognoses as it has been found in various studies that once radiographic diagnosis of osteonecrosis is made, collapse usually occurs within 2 years in 32–79 % of patients (18,23,24).

2.2.2. - Magnetic resonance imaging (MRI)

Imaging by magnetic resonance is considered the method of choice for detecting and staging ONFH due to its multiplanar imaging, outstanding soft tissue contrast, and the ability to discriminate fat from other bone marrow tissues (25–27). Compared to other imaging methods such as plain radiography or computed tomography (CT) magnetic resonance imaging (MRI) has been found to have the highest sensitivity and specificity (27,28) . It is a practical tool for early diagnosing (Figure 2), quantification of the extent of the lesion within the femoral head and for its further stratification (22,29).

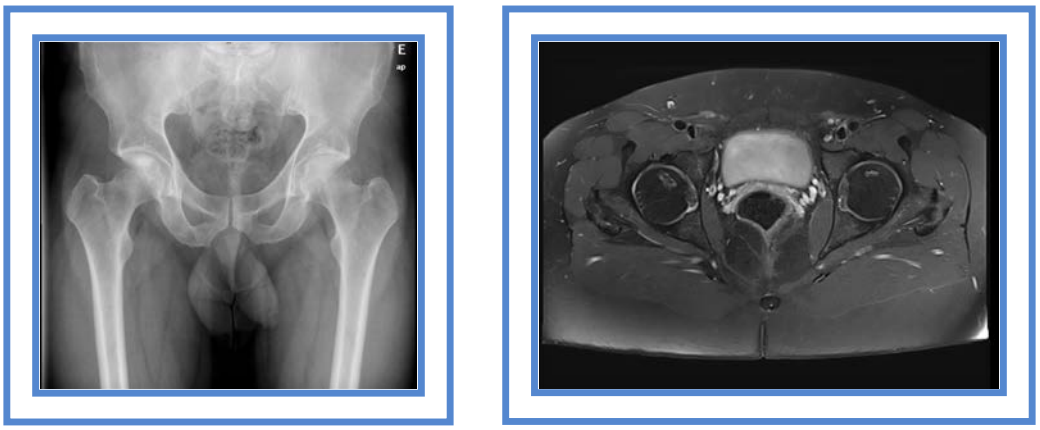


Figure 2: Patient with bilateral ONFH, the AP pelvis radiograph shows no signs of ONFH in the left hip. An axial pelvis MRI in T2 sequence shows a small lesion corresponding to a grade I ONFH.

A circumscribed subchondral "band-like" lesion with low signal intensity on T1-w images is pathognomonic of ONFH (30). This finding does not depend on normal or ONFH-suggesting radiographs. The "double-line" sign is seen on T2-w Spin Echo or Turbo Spin Echo sequences and consists of a low signal intensity outer rim and a high signal intensity inner rim. This sign was introduced by Mitchell *et al.* in 1987 and was considered pathognomonic for ONFH since the outer rim represents the reactive bone; the inner rim represents the vascular and repair tissue at the necrotic-viable osseous interface. The double-line sign was present in 80% of the lesions but no radiograph correlation was performed. The region within the "double-line" sign may demonstrate hypo, iso and hyperintensity when compared to normal marrow.

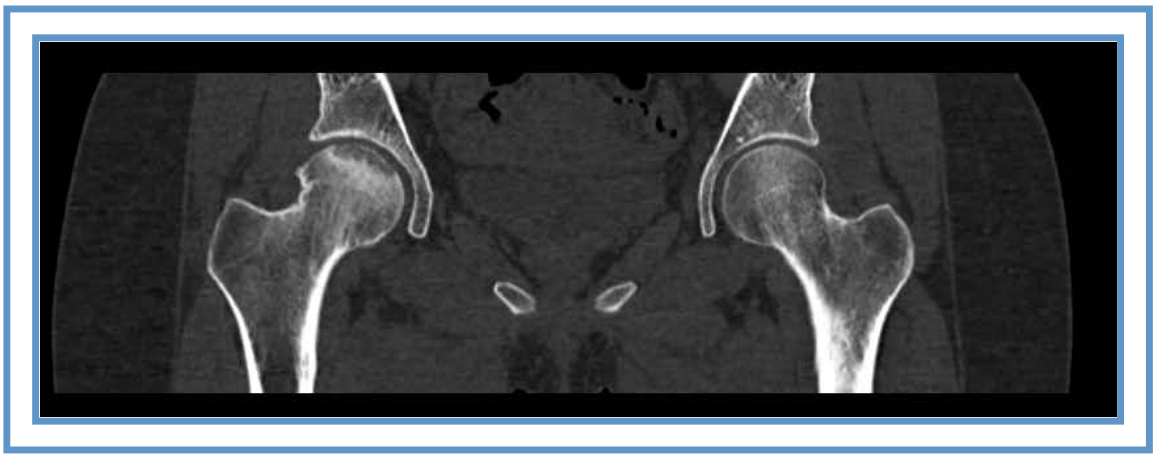
MRI offers many advantages over other imaging methods. First, it does not use ionizing radiation, which is very important when managing younger patients, particularly those in their growing periods. It also allows assessing different planes

(axial, sagittal, coronal, medium planes or combinations of planes). The resolution is superior in soft tissue and has a high spatial resolution and contrast.

Magnetic resonance imaging (MRI) is costlier but is superior in detecting early stages of the disease because of its higher sensibility and specificity and is considered the imaging method of choice compared to plain radiographs, computed tomography, or scintigraphy (30). It is useful for early diagnosis, quantitative evaluation of disease extension within the femoral head, and for staging of the disease (29).

2.2.3. - Computed tomography (CT)

Computerized tomography (CT) is considered the most sensitive diagnostic tool for the detection of subchondral fractures (Figure 3) of the femoral head as it clearly delineates the outline of the subchondral bone (31) . In order to determine the best plane to see a subchondral fracture will depend upon the orientation of the necrotic segment. This method is also a practical way to corroborate small areas of collapse that are not evident on plain radiographs or MRI. This method offers the disadvantage of having to have patients exposed to radiation.



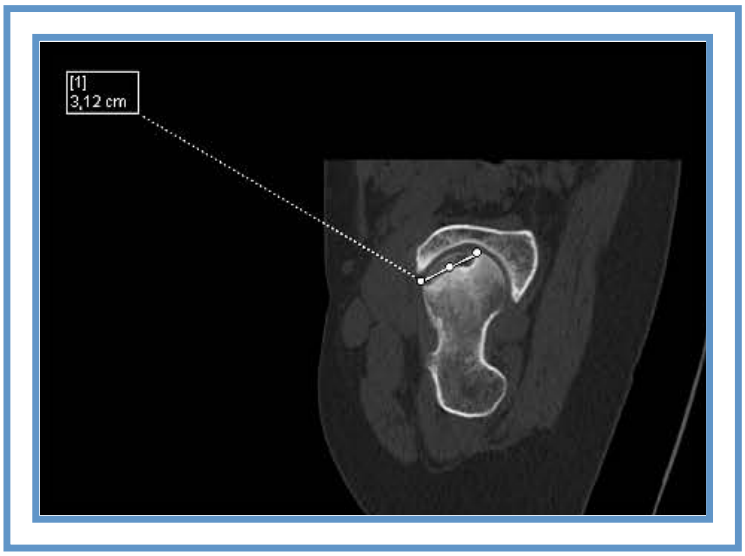


Figure 3: An AP and sagittal view of a pelvis CT scan showing a subchondral fracture and collapsed femoral head.

Table 2: Differential diagnoses of OFNH by particular imaging methods (modified from Castiglioni (10).

Radiography	Computerized tomography	Magnetic Resonance Imaging
Metastasis	Osteoarthritis	Edema
Osteomyelitis	Stress Fracture	Osteoporosis transient
Transitory osteoporosis	Infection	Bone Contusion
Bone sarcoma	Myeloma plasmocytic	Stress fracture
Stress fracture	Metastasis	Infection
Epiphyseal Dysplasia	Metastasis	Arthritis

2.2.4. - Bone scanning

Bone scanning is another method for assessing ONFH. As necrotic bone tissue does not take up the technetium-99 isotope, in the scan it appears as a "cold" structure, while the surrounding remodeling tissue would appear as "hot" zones. Early stages of the disease are therefore characterized by "cold within hot" regions. In spite of its advantages for detecting multifocal lesions, bone scanning is less specific which offers difficulties when making differential diagnoses. It also offers poor spatial resolution and is not a quantitative tool which is why it is not a preferred tool for managing ONFH (32).

2.2.5 Positron emission tomography (PET) scan.

Recent studies have investigated the role of positron emission tomography (PET-CT-F18) in the diagnosis of ONFH (Figure 4). It is based on the use of radioactively labeled fluorine 18 and the high level of glucose metabolism of the inflammatory cells that are present in the ONFH. Dasa *et al.* in a comparative study between PET, scintigraphy and MRI they found that PET was more sensitive in the early diagnosis of ONFH (33).

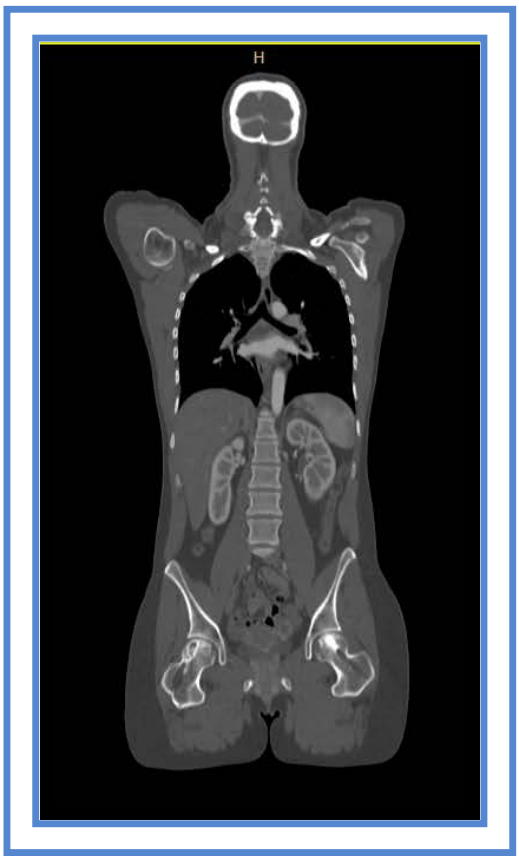


Figure 4: PET scan image in a patient with bilateral hip ONFH.

3. - Classification systems of ONFH

Based on clinical and radiological features of osteonecrosis different staging systems have been developed. In 2006, Mont *et al.* published a systematic review of the literature where they reported the 16 major systems used to classify osteonecrosis (34). Out of all them, the most widely used appears to be the Ficat classification which accounted for more than 63% of the reported studies (15). Other three widely spread classification systems were the University of Pennsylvania System (20%) (28), the Association Research Circulation Osseous (ARCO) system (12%) (34) and the Japanese Orthopaedic Association System (5%) (35).

3.1. - Ficat Classification

Ficat and Arlet developed this classification system in the 1960s. It originally had three stages and it has been modified at least four times. In the present day, most physicians will refer to the four-stage system that is rather simple.

Table 3: ONFH Ficat Classification

STAGE	PAIN	X RAY	MRI
I	+	Normal	+
II	+	Diffuse sclerosis, cysts	+
III	+	Subchondral fracture (crescent sign; with or without head collapse)	+
IV	+	Femoral head collapse, acetabular involvement, and joint destruction (osteoarthritis)	+

3.2. - ARCO Classification

The Association Research Circulation Osseous (ARO) classification system is more practical from a clinical point of view as it allows, not only to establish the prognosis and follow-up of disease progression but also to establish the best line of therapy.

Table 4: ONFH ARCO Classification.

STAGE	X RAY	MRI	LOCATION	EXTENSION
0	Normal	−	No	No
I	Normal	+	Medial Central Lateral	Mild (<15% femoral head) Moderate (15-30% femoral head) Severe (> 30% femoral head)
II	Sclerosis, osteolysis, focal porosis	+		
III	Crescent sign" and/or flattening of the articular surface	+		
IV	Osteoarthritis	+	No	No

3.3. - Radiographic Classification System of the Japanese Orthopedic Association

The radiographic classification system of the Japanese Orthopedic Association added to its classification location of the lesion and stratification of the disease, though several authors have argued that location of the lesion is not as important as its size and suggest it not be adopted universally (36,37).

Stage	Nuclear Magnetic Resonance Imaging (MRI)
I	Presence of demarcation line in the femoral head
IA	Outermost demarcation line is located on the middle third of the bearing surface
IB	Outermost demarcation line is located in the central third of the bearing surface
IC	Outermost demarcation line is located on the lateral third
II	Flattening of the femoral head on the abutment surface without demarcation line
III	Cystic radiolucent lesions without demarcation lines
IIIA	Cystic lesions located anteriorly and medially in the femoral head, away from the support zone
IIIB	Cystic lesions located in the lateral area of the support surface

3.4. - University of Pennsylvania Classification and Staging System

The two most important characteristics of the University of Pennsylvania Classification System are: inclusion of MRI findings and lesion size, which are now recognized to be essential in any effective classification system.

Stage		Findings
0		Normal or nondiagnostic radiograph, bone scan, and MRI
I		Normal radiograph; abnormal bone scan and/or MRI
	A	Mild (<15% of head affected)
	B	Moderate (15 to 30%)
	C	Severe (>30%)
II		Lucent and sclerotic changes in femoral head
	A	Mild (<15%)
	B	Moderate (15 to 30%)
	C	Severe (>30%)
III		Subchondral collapse (crescent sign) without flattening
	A	Mild (> 15% of articular surface)
	B	Moderate (15 to 30%)
	C	Severe (> 30%)

IV Flattening of femoral head.

A Mild (<15% of the surface and <2mm of depression)

B Moderate (15-30% of the surface or 2-4mm of depression)

C Severe (> 30% of the surface or> 4mm of depression)

V Joint narrowing and/or acetabular changes

A Mild

B Moderate

C Severe

Stage VI Advanced degenerative changes.

There are many similarities across the classifications used to stratify ONFH. Magnetic resonance imaging and plain radiography are essential tools that allow such classifications. According to Mont et al the following parameters have been found to be the most useful: (1) presence or absence of collapse; (2) size of lesion; (3) head depression; (4) acetabular involvement; (5) crescent sign; and (6) diffuse sclerosis and the presence of cysts (38).

3.5. - Steinberg Classification

Steinberg's classification system is based on the radiographic appearance and location of lesion. It primarily differs from the other systems in that it quantifies femoral head involvement, which allows direct comparison between series. Seven stages of involvement are identified. Following staging, extent of involvement of femoral head is recorded as mild, moderate or severe (22).

Table 5: ONFH Steinberg Classification.

STAGE	X RAY	MRI	EXTENSION
0	Normal	−	No
I	Normal	+	Mild (<15% femoral head)
II	Cystic and sclerosis	+	Moderate (15-30% femoral head)
III	Crescent sign	+	Severe (> 30% femoral head)
IV	Flattening of femoral head	+	
V	Joint space narrowing	+	No
VI	Advanced degenerative changes	+	No

4. - Pathogenesis

As mentioned earlier, osteonecrosis of the femoral head (ONFH) is a multifactorial process; and whichever the underlying event is, it consistently derives in the disruption of blood supply to the affected bone area, which eventually leads to ischemia and necrosis. It is important to correctly recognize the pathogenic pathway underlying any case of ONFH in order to establish the most successful line of

therapy. We will address the main pathogenic pathways that may result in the obstruction of the blood supply to the femoral head. However the pathogenesis of the non-traumatic ONFH remains unclear.

~ *Vascular occlusion*

It is important that we briefly review the vascular anatomy of the femoral head for better understanding of the pathogenesis of OFNH. The femoral head is provided of blood by the arteries that arise mainly from the median femoral circumflex artery (MFCA) and the inferior gluteal artery (IGA). There are lesser contributing vessels that arise from the lateral femoral circumflex artery (LFCA), obturator artery, superior gluteal artery and first perforating branch of the deep femoral artery.

The MFCA forms the lateral epiphyseal arteries that penetrate the femoral head through its posterosuperior phase. Inside the cortex of the femoral head, these vessels run in a medial and anterior fashion and reach the anterosuperior quadrant of the femoral head. They account for around 80% of the blood supply of the femoral epiphysis (39) (40). It appears that the anatomic predisposition of necrosis to affect more often the anterosuperior quadrant of the femoral head is due in part to the intracortical obstruction of these vessels (41).

The blood supply of the femoral head is very particular given that the irrigation patterns remain the same at maturity and do not change over the course of the life of an individual (39).

Kikkawa *et al.* were able to demonstrate through a rat model of ONFH that resembles Legg-Calvé-Perthes disease (LCPD) in children, an altered expression of insulin-like growth factor 1 (IGF-1) (42). Aberrant expression of IGF-1 downregulates the expression of type X collagen during ossification of the epiphyses and consequently, the femoral epiphyseal plate suffers mechanical instability. These authors postulate that the physical interruption of blood supply caused by this unsteady sector causes ischemia in a way similar to necrosis induced by traumatic causes.

~ *Abnormal lipid metabolism and fat embolism*

It has been established that hyperlipidemic states following the use of steroids makes the amount of fat inside the femoral head larger, which makes intracortical pressure to rise and consequently lead to the collapse of the sinusoids (43).

Steroid-treated rabbit studies performed by Wang *et al.* showed that femoral head adipocytes increased in size by 25% when compared to untreated rabbits (44). Other studies ensuing this same line of research demonstrated that there was a clear correlation between fat cell size and increased intracortical pressure, with decreased blood flow (45). When treated animals received antilipemic treatment with clofibrate, a decrease in adipocyte size and femoral pressure was observed, as well as an increase of the blood flow (46).

Studies commanded by different authors raised questions about the role of fat emboli in the development of ONFH as one of them had shown that rabbits developed osteonecrosis when given arterial injections of lipids (47). Previous

studies by Wang had demonstrated that rabbits receiving large doses of cortisone during the growing or adult phases, besides from displaying altered lipid metabolisms (increased serum cholesterol, hepatic fatty metamorphosis); its tissue sections revealed fat emboli that partially obliterated the microcirculation of the subchondral vessels of both femoral and humeral heads (44). These studies led to the yet unproven theory that the emboli that block the microvasculature might lead to an activation of the complement pathway with deposition of immune complexes that lead to hemorrhage and osteonecrosis.

Other authors suggest that the increase of fat content may result in augmented intracortical pressure as a cause of osteonecrosis. As with other causes of osteonecrosis, a disruption of the blood supply is the final pathway, which may well be due to this phenomenon (48).

~ *Intravascular coagulation (IC)*

Research studies in the 1990s demonstrated that both thrombophilia and hypofibrinolysis are associated with the development of osteonecrosis (49,50). More recently, abnormalities of the nitric oxide metabolism mediated by eNOS have also been acknowledged as an important player in the pathogenesis of osteonecrosis (51,52).

These authors believe that these conditions account for several cases of idiopathic osteonecrosis. They observed that patients repeatedly referred with idiopathic osteonecrosis, were more likely to have familial thrombophilia which included heterozygosity for the factor V Leiden mutation and high levels of

homocysteine, factor VIII and anticardiolipin IgM antibody, when they were compared to the control group (51,52).

As we have already made clear, many factors may play a role in the pathogenesis of osteonecrosis. It has now been postulated that the series of events in the pathogenesis of ONFH would be as follows: venous thrombi due to thrombophilia-hypofibrinolysis obstruct the venous outflow which would, in turn, lead to an increase of the intracortical venous pressure, a decrease of the arterial blood flow, ischemia and cell death (53,54). Some experimental models have confirmed that the initial event is in fact, venal occlusion (55).

~ *Intraosseous pressure*

High-pressure levels inside the femoral head can also obstruct blood flow. The femoral head is a rigid sphere-like structure that contains porous bone tissue, marrow and fat. Should there be an increase in any of these contained elements, whether for reparation of bone tissue or any other process, that pressure inside the femoral head would increase and very likely, interrupt normal blood flow (48,56).

~ *Mechanical strain*

Research studies on rabbits performed by Iwasaki *et al.* in which the animals with occlusion of the lateral epiphyseal arteries developed osteonecrosis; also showed that animals in the same condition but with their sciatic nerve cut (thus preventing the bearing of weight) developed osteonecrosis less frequently (57). This lead to the conclusion that mechanical strain may play an important role in the pathogenesis of non-traumatic osteonecrosis.

Other studies in rats supported this hypothesis. The animals were forced to stand on their hind limbs in order to be able to feed. The study group of rats showed higher prevalence of osteonecrosis (33.3%) that would suggest mechanical stress participates in the etiology of ONFH (58).

5. - Clinical entities commonly associated to ONFH

The main factors that contribute to the development of osteonecrosis are:

Corticoid use:

In 1932, Cushing presented the adverse effects on bone that derived from sustained high levels of cortisol. Once cortisone was approved to treat rheumatoid arthritis, physicians immediately began to notice the deleterious effects on skeletal tissue of glucocorticoid administration. Treatment with cortisone, prednisolone, and prednisone clearly generated skeletal complications, such as osteoporosis and fractures (59). Soon afterwards, studies began to show that high-dose steroid therapy lead to the collapse of the femoral head (60).

Currently, it is widely known that steroid therapy is one of the most usual causes of secondary osteoporosis and one of the primary causes of non-traumatic osteonecrosis. Patients who receive glucocorticoids long-term, show fractures in 30–50% of the cases and ONFH in 9–40% of the cases (61,62).

Weinstein *et al.* widely studied the impact of steroids on osteocyte activity in a mouse model (63). When the experimental animals received high doses of

prednisolone for almost a month, an increase in both osteoblast and osteoclast apoptosis at the cortical bone was observed. These changes included a decrease in bone density, formation and turnover. Histological tissue sections showed increased production of porous bone and a reduction of trabecular width along with low levels of serum osteocalcin.

As was also seen in mice studies, patients who exhibited glucocorticoid-induced osteoporosis also had an increase in osteoblast and osteocyte apoptosis. The decrease in osteoclast production would help explain the reduction in bone turnover while the decrease of osteoblasts would clarify the decline in bone formation and reduction of the trabecular space. Moreover, apoptotic *debris* may also contribute such congested conditions. The evidence thus points towards changes in the number of bone cells as being the cause of steroid-induced ONFH (63).

Another mechanism by which corticoids produce osteonecrosis is by their activity upon the endothelial cells of the femoral vessels. High doses of corticosteroids inhibit fibrinolytic activity thus disturbing the coagulation pathway. Moreover, as the angiogenesis process is altered a reduction of vascular endothelial growth factor (VEGF) synthesis is observed as well (64). Endothelial cell apoptosis further encourages clot formation and platelet activation by the binding of thrombocytes to the endothelium (65).

Alcohol:

Excessive alcohol consumption of alcohol is a well-established risk factor for the development of ONFH, although its etiology is not quite totally clear. Rico *et al.* studied the incidence of alcohol-associated ONFH in 57 patients and found an incidence of ONFH of 29% and 12% of idiopathic osteonecrosis (66).

Another study was conducted on a larger number of patients with non-traumatic osteonecrosis of the femoral head three without history of systemic steroid use. They found a significant elevated risk for regular drinkers (RR = 7.8, $p < 0.001$) and a relationship that was clearly dose-dependent as patients who consumed less than 400 ml/week of alcohol showed lower relative odds of developing ONFH (RR = 3.3) than those who consumed 400-1000 ml/week (RR = 9.8) and even less so than those whose alcohol consumption was over 1000 ml/week (RR = 7.9) (67).

Wang *et al.* investigated the effect of alcohol on rabbit bone marrow and on the differentiation of mouse bone marrow stromal cells (68). They found that alcohol caused an increase in serum triglycerides and cholesterol, as well as a reduction of the activity of the superoxide dismutase. Histological examination of bone marrow revealed adipogenesis and fatty infiltration in hepatic tissue. As for the femoral head, microscopic studies showed hypertrophy and proliferation of adipocytes and a decrease in hematopoiesis. The control group showed no evidence of any of these manifestations. Wang *et al.* conclude that alcohol directly induces adipogenesis, decreases osteogenesis in bone marrow stroma, and produces intracellular lipid deposits resulting in the death of osteocytes, which may be associated with the

development of osteonecrosis, especially in patients with long-term and excessive use of alcohol.

Tobacco:

A paper published in 1993, investigated the association of cigarette smoking, among other variables, with the development of idiopathic osteonecrosis of the femoral head. The research comprised a nationwide multicenter case-control study conducted in Japan during 1988-1990, comparing 118 cases with no history of systemic corticosteroid use with 236 controls matched for sex, age, ethnicity, clinic, and date of initial examination. These researchers found an increased risk for current smokers (relative odds = 4.7), but the cumulative effect of smoking was not apparent at 20 pack-years or over (69).

A More recent study by Wen *et al.* found that compared to non-smokers, current smokers had a higher risk of developing ONFH showing odds ratios of 2.5; as well as former smokers who displayed OR of 1.82 (70). The current smokers were further classified as heavy (>20 cigarettes/day) and light smokers (<20 cigarettes/day). Both demonstrated higher risk of ONFH than non-smokers. When the patients were categorized by pack-years to evaluate the impact of time of exposure to tobacco smoke, the heavy smokers (>20 packs-years) showed higher risk than the non-smoker group but this was not the case for the light smokers (<20 packs-year) that displayed no significant greater risk than non-smokers. They concluded that these findings suggest that current smokers are at a higher risk of ONFH, which persists even after having quit and that heavy cigarette smoking confers a higher risk of ONFH than light smoking (70).

Hemoglobinopathies:

Patients with sickle cell hemoglobinopathies are often complicated with osteonecrosis of the femoral and humeral heads due to the characteristic vaso-occlusive events of this ailment; and is generally bilateral in nature (71,72).

Milner *et al.* found that patients who carry both alleles for hemoglobin SS had the highest incidence of sickle-cell associated osteonecrosis (73). Osteonecrosis in patients with drepanocytosis is estimated to prevail in 3 to 5% of them, while asymptomatic osteonecrosis is more widespread (10-41%) (24,73).

Osteonecrosis of the femoral head may progress to collapse of the head and persistent joint pain in patients with sickle cell disease (74). Advanced ONFH accounts for most of total hip arthroplasties performed in young patients with drepanocytosis (74,75). As there is no standard medical management of ONFH associated to SCD, recommendations of symptomatic management with NSAIDs, physiotherapy and early hip surgery have been published (76).

More recently, Adesina *et al.* used California's Office of Statewide Planning and Development discharge databases (1991-2013) to identify and estimate factors associated with ONFH diagnosis (77). The cumulative incidence of ONFH to age 30 years was higher among patients with more severe sickle cell disease (24%; vs 8% in less severe). From 2003 to 2013, patients with more severe cases of SCD were more likely to develop ONFH. Twenty-seven percent of post–hip surgery patients was readmitted within 30 days, mostly due to painful vaso-occlusive crises. ONFH is a common SCD complication that increases with age; ongoing studies into prevention

and effective nonsurgical interventions for SCD-induced osteonecrosis must remain a high research priority.

Thrombophilia and coagulopathy:

As we have stated earlier, hypercoagulable states such as thrombophilia and hypofibrinolysis, are often a cause of ONFH (49,50).

More recent studies conducted by Glueck *et al.* in which they compared measures of thrombophilia and hypofibrinolysis in subjects with idiopathic and secondary (corticosteroid-induced) osteonecrosis of the femoral head. These measures included the Factor V Leiden mutation, resistance to activated protein C, Factor VIII and hypofibrinolytic ($Lp_{[a]}$) (52). Their results revealed that heritable thrombophilia and hypofibrinolysis were more common in patients with osteonecrosis ($p = 0.0004$); and that patients with idiopathic osteonecrosis were more likely to have high levels of heritable thrombophilic Factor VIII and more likely to have inherited high levels of hypofibrinolytic Lp(a). Moreover, patients with secondary osteonecrosis had a higher chance of having high levels of Factor VIII (>150%), of being heterozygous for the Factor V Leiden mutation and to have thrombophilic resistance to protein C.

Treatment with low-molecular weight heparin has been shown to result in the amelioration of osteonecrosis (78), which led these and other authors to suggest that thrombophilia-hypofibrinolysis-mediated thrombosis is a potentially reversible cause of ONFH (79,80).

Primary antiphospholipid syndrome:

Antiphospholipid syndrome (APS) is an autoimmune disorder with clinical manifestations of multiple thrombotic episodes, fetal loses and high levels of antiphospholipid antibodies, including lupus anticoagulant antibodies (LAC), anti-cardiolipin antibodies (aCL), and β_2-glycoprotein I antibodies (81,82).

The occurrence of thrombotic microangiopathy (small-vessel occlusion) associated with anti-phospholipid antibodies (aPL) has been well documented in wide variety of target organs.

In vitro studies have demonstrated that when endothelial cells are incubated with aPL and β_2-glycoprotein I (β2-GPI), they begin to express significantly higher levels of adhesion molecules such as ICAM-1 (intercellular cell adhesion molecule 1), VCAM-1 (vascular cell-adhesion molecule 1) and E-selectine (83). The antibodies, aPL and β2-GPI, also induce platelet activation. These effects are all amplified by the activation of the complement (84).

It has been well established that aCL are associated with thrombosis of a variety of vessels occurring in many different sites and bone irrigation is not exempt of enduring ischemia due to this phenomenon (85). Using magnetic resonance imaging, Tektonidou el al were able to demonstrate that 20% of patients with primary antiphospholipid syndrome (PAPS) exhibited microthrombi in the microcirculatory system of the femoral head (86)

Despite the fact that osteonecrosis of the femoral head is a rare manifestation of PAPS, it should never be overlooked as a differential diagnosis in patients with mechanical joint pain and no other risk factors associated (87).

Radiation-induced osteonecrosis (osteoradionecrosis):

The treatment of a wide variety of neoplastic disorders often requires radiation therapy as part of its management. However, its use may induce changes in bone structures. These induced changes greatly depend on the age of the patient, the absorbed dose, the size of the field, beam energy and fractionation (88). In the development of skeletal structures, growth disturbances are greater in younger patients, independently of the dose received.

In patients with mature skeletal systems, radiation therapy results in impairment of osteoblast function, which means a decrease in matrix production. Its radiographical manifestations typically occur one year after having received therapy and usually includes images of osteopenia (89). Vascular damage may also contribute to the late manifestations of bone atrophy induced by radiation. Bone repair derives in the deposition of new bone upon the necrotic trabecular bone tissue. At this stage, radiographs would reveal heterogeneous bone density with punctate areas of increased density, osteopenia, and coarse trabeculation (90). The weakened bone shows more propensity to fractures and the array of radiographic findings is known as radiation osteitis or osteoradionecrosis.

A study conducted on older women concluded that women, who underwent radiation therapy for different types of pelvic cancer, were more likely to suffer

fractures than women that had not been irradiated. These results were statistically significative and most fractures (90%) occurred at the hip (91).

The incidence of osteonecrosis in post-radiation therapy of the femoral head has been reported to be 4 in 763 patients (92). Treatments that include chemotherapy as well may further increase the risk of osteonecrosis in patients who received radiation therapy.

Alterations of the vascular anatomy:

Although not frequently, altered vascular anatomic patterns have been detected among patients with osteonecrosis of the femoral head, especially hypoplasia of the anterior capsular artery, which is the most common. The lateral epiphyseal vessels are usually the ones that deteriorate before, and consequently this area is where necrosis of the femoral head most frequently develops (93).

Pregnancy:

Although not a commonly associated factor, late pregnancy is a cause of hip pain that may occasionally be triggered by ONFH, especially in women with smaller skeletal structures and larger pregnancy weight gain (94). Mechanical stress resulting from the weight acquired during pregnancy might enhance the chances for developing ONFH.

Pregnancy associated-ONFH is commonly misdiagnosed as transient osteoporosis of the hip. Careful assessment of MRI with findings of a double-density signal or evidence of progression of the disease in plain radiographs is essential for a

correct diagnosis. High suspicion levels may lead to earlier diagnoses and prognoses for women in danger of developing ONFH associated with pregnancy.

Another risk factor within this group may be the hypercoagulability state and venous congestion that characterize late pregnancy. There are greater chances of developing ONFH on the left side due to greater tendency of the left common iliac vein to be compressed by the fetus and develop deep venous thrombosis (95).

HIV:

Patients who have acquired human immunodeficiency virus (HIV) are at increased risk for developing ONFH (96,97). It is not clear whether the virus is the direct cause, or if the treatments used for management of the disease (retroviral agents such as protease inhibitors, corticosteroids, etc.) are the etiologic factors at play. Blacksin et al found that ONFH was not directly related to the HIV but rather to the use of corticosteroids (96). Miller *et al.* conducted a study where they found that 4.4% of HIV-infected adults suffered from ONFH and no evident hip lesions; such was not the case for the age and gender-matched control group. Hip lesions were more likely to occur in patients who received corticosteroids, lipid-lowering drugs, or testosterone (97). Although many studies have attributed the pathogenesis retroviral therapy itself, studies by Ries *et al.* have identified patients with ONFH and no other associated risk factors, which would suggest that the HIV infection is in itself a risk factor for developing the condition (98).

Gaucher disease:

Gaucher disease is the most prevalent inherited, lysosomal disorder (99). It is caused by a genetic deficiency in the activity of the membrane-bound beta-glucocerebrosidase enzyme, responsible for the degradation of glucocerebroside, a sphingolipid. The deficiency derives in an accumulation of glucocerebroside in lysosomes of monocytes and macrophages which are called "Gaucher cells" when this phenomenon of fat engorgement occurs.

In patients with Gaucher disease, osteonecrosis commonly develops in the long bones and vertebrae and is probably the most clinically significant and disabling skeletal manifestation of the disease (100). It has been found that male gender and antecedents of splenectomy are independent risk factors for developing ONFH in patients with the condition.

Genetics:

Some studies have provided evidence that support the role of genetic factors in the development of osteonecrosis. This suggests that mutations might be involved in the pathogenesis of the disease.

Some aforementioned disorders commonly associated with osteonecrosis display genetic inheritance such as sickle cell anemia, thrombophilia.

In 2004, Chen *et al.* identified two Taiwanese families (4 generations) who displayed autosomal dominant inheritance of ONFH. They were able to exclude linkage with elements implicated in thrombophilia or hypofibrinolysis (PROC,

PROS1, PAI) (101). Genome-wide studies performed by these authors found a marker on chromosome 12 (12q13, D12S368). In later studies, Liu et al (2005) identified three families that exhibited autosomal dominant inheritance of ONFH that mapped out for the 12q13 phenotype (102). Haplotype analyses of the families were performed in search for candidate genes in this critical chromosomic region. After promoter and exonic sequencing of the *type II collagen gene* (COL2A1) from patients with sporadic or familial ONFH, they were able to determine same mutation (gly1170ser) in two of the families and another mutation if the COL2A1 in another family (gly717ser). Such mutations did not occur in patients with sporadic cases of the disease. The authors suggest that genetic studies of the COL2A1 gene might lead to early diagnosis and management of the disease.

Genetic findings associated to the development of osteonecrosis hold the promise not only of further elucidating the pathogenesis of osteonecrosis but for the identification of high-risk patients.

6. - Management of ONFH

Treatment of osteonecrosis of the femoral head will usually depend on many factors. Management of the condition is grossly divided into surgical and nonoperative methods.

6.1. - Nonsurgical management of ONFH

Most nonoperative treatments involve the restriction of weight bearing by the use of canes, crutches or walkers with the intention of slowing down disease progression so that procedures for preservation of the femoral head can be later carried out.

Mont *et al.* published a meta-analysis of the results of protected weight-bearing in more than 800 patients and demonstrated that four years after the diagnosis was established, more than 80% of the showed progression towards head collapse and arthritis (103). Ultimately, the majority of the patients required total hip replacement that led the authors to conclude that osteonecrosis of the femoral head should not be treated in a conservative manner.

In patients in whom the disease is diagnosed early and no collapse of the head has occurred, it is more likely that newer noninvasive interventions may be applied; however, this would require standardization of treatment modalities for each stage, which has yet to be established.

In the case of asymptomatic pre-collapse injuries, observation is essential, and many reports now justify nonsurgical methods for these kind of lesions as they have shown to follow a better natural history (103–106). Usually, these lesions are found in the contralateral hip when the patient is being evaluated for the symptomatic hip.

Cheng *et al.* provided evidence that some small lesions may heal spontaneously after having studied 30 patients with hip disorders where three of them exhibited such phenomenon (107). They found that small, asymptomatic lesions in early stages of the disease were more likely to resolve spontaneously.

Pharmacological management of ONFH

Pharmacological treatment of ONFH should be addressed to specific risk factors associated to the condition. In this manner, lipid-lowering agents should be used when there is suspicion of lipid emboli or adipocyte hypertrophy; anticoagulants for preventing venous thrombosis and vasodilators for situations of increased intraosseous pressure and bone resorption.

Lipid-lowering treatments

Studies by Wang *et al.* (1997) on chickens treated with lovastatin to prevent steroid-induced adipogenesis have given way to treating systemic lupus erythematosus (SLE) patients with lipid-lowering drugs. Based on these studies, it has been suggested that osteonecrosis in patients with SLE may arise as a result of shifting of mesenchymal stem cells towards an adipocytic lineage instead of osteoblastic. Thus, lipid-lowering treatment may serve to divert normal osteoblastic differentiation.

Anticoagulants

Research by Glueck *et al.* on patients with hypofibrinolysis associated with high activity levels of plasminogen activator or serum lipoprotein treated with stanozolol, an anabolic steroid (108). After one year of treatment, patients exhibited

less symptomatology. In another study, Glueck *et al.* administered enoxaparin (60 mg/day for 12 weeks) to patients with hypofibrinolytic or thrombophilic disorders and early stages of ONFH. After two years, patients showed stable Ficat and Arlet stage I or stage II disease by radiograph assessment, and 89% did not require hip surgery (78).

Vasodilators

The vasodilator agent Iloprost, a prostacyclin derivative, has been evaluated for the treatment of ONFH and bone marrow edema syndrome (109). In this study they evaluated 17 patients with early-stage osteonecrosis of the femoral head and after one year of iloprost treatment, all patients had clinical and radiological improvement.

Biphosphonates

Biosphophonates are pharmaceutical agents that inhibit osteoclast activity thus restraining the resorption of bone that may in theory, slow progression of ONFH given the hypothesis that increased bone resorption is a contributing factor to collapse of the femoral head.

Diverse experimental studies performed on different animals have demonstrated that treatment with these agents reduces the prevalence of femoral head collapse (110–112). Some clinical reports have proposed that alendronate seems to be beneficial for patients with ONFH. Agarwala *et al.* published a study conducted on patients treated with this agent (10 mg/day) where they found clinical

improvement and reduction on disability scores and only six patients of 60 required surgery (113).

6.2. - Biophysical management of ONFH

Different methods have been used to treat ONFH in a noninvasive manner. Electromagnetic stimulation extracorporeal shock-wave therapy (114), and hyperbaric oxygen (115) are some examples.

Despite showing some effectiveness in studies conducted during the 1980s, electromagnetic stimulation for treatment of ONFH has not been approved by the Food and Drug Administration (FDA).

Wang *et al.* compared the results of shock-wave therapy in twenty-three patients with those of a group treated with nonvascularized fibular grafting. At a mean of twenty-five months, 79% of the shock-wave group had improved Harris hip scores compared with 29% of the group treated with nonvascularized fibular grafting (116).

The use of hyperbaric oxygen has had more controversial results and patients find it costly and time-consuming. Studies performed by Peskin *et al.* used hyperbaric oxygen for preventing femoral head collapse in a rat model of vascular deprivation-induced osteonecrosis (115). Another study analyzed sixteen hips pertaining to twelve patients who had early-stage ONFH who were treated with hyperbaric oxygen for 100 days. Thirteen of the sixteen hips showed improvement of MRI features of the disease (117).

6.2. - Surgical management of ONFH

Surgical treatment of osteonecrosis can be broadly divided into femoral head–preserving procedures and hip arthroplasty. Femoral head-preserving techniques include core decompression with or without supplemental nonvascularized bone grafting, vascularized bone grafting, concentrated stem cells, biologic adjuncts, and osteotomies. Hip arthroplasty procedures include total hip arthroplasty (THA) and resurfacing arthroplasty. However, the ideal surgical treatment has not been defined and decision of treatment should include individualization of the patients' disease and potential risk factors.

Femoral head-preserving techniques

- *Core decompression*

Core decompression is a widespread treatment tool for early-stage ONFH as it is a very simple procedure whether performed with autologous bone grafting or not. Its goal is to reduce intraosseous pressure and restore vascular flow within the femoral head through a drill hole in the distal end of the greater trochanter that instantly and dramatically reduces pain (Figure 5).

Small lesions at a pre-collapse stage show better outcomes when treated with core decompression. A retrospective study conducted by Israelite *et al.* using core decompression with bone grafting in 276 hips (minimun follow-up of two years) showed that 38% of the patients required total hip arthroplasty (THA) (118). In precollapse stages, these smaller lesions (15% of the femoral head) had a significantly better outcome (14% required arthroplasty) compared with intermediate

lesions (15% to 30% of the femoral head, 48% arthroplasty) and large lesions (30% of the femoral head; 42% arthroplasty).

Marker *et al.* published a systematic review showing that since 1992 of 1,268 hips treated with core decompression, 70% did not require any additional surgery and 63% had successful radiographic outcomes (119).

Figure 5: An intraoperative core decompression technique image.

Core decompression with nonvascularized grafts, stem cells or biologic adjuncts

The core decompression technique can be supplemented with nonvascularized autografts (allograft bone or demineralized bone matrix) to provide mechanical support of the affected joint and thus, delay the need for more invasive procedures, such as arthroplasty.

The non-vascularised cortical graft was originally popularised by Phemister (120). However, it is not widely observed as a treatment technique nowadays. There are various grafting techniques depending on the entry; through the core tract (Phemister technique), through a cortical window at the junction of the cartilage and the femoral neck (light bulb technique) or through a cartilage window (trap door technique).

Patients with osteonecrosis display a decreased ability of mesenchymal cells for osteogenic differentiation (121). For this reason, recent studies have now shifted their attention towards the introduction of bone morphogenetic proteins (BMPs) or bone marrow cells for enhancement of bone formation and repairing processes when treating larger lesions (3,122,123).

In 2004, Lieberman *et al.* published a study conducted on 15 patients (a total of 11 hips) treated with core decompression, autogenous bone graft, and a fibular allograft perfused with human BMP and non-collagenous proteins (3). At a mean follow-up of 53 months, 93% of hips with Ficat-Arlet stage IIA disease had relief of pain and no radiological findings of disease progression.

Calori *et al.* performed a retrospective clinical study to determine the efficacy of combined core decompression techniques on clinical outcomes and progression ONFH (124). The core decompression technique was combined with either recombinant bone morphogenetic proteins (BMPs), autologous mesenchymal stem cells (MSCs) and xenograft bone substitute; and then placed into the necrotic region of the femoral head. A total of 38 patients (40 hips) with early stage osteonecrosis of

the femoral head were studied over a 4-year period (seven hips, Ficat I; 25 hips, Ficat II; eight hips, Ficat III). Clinical and radiographic healing occurred in 33 of 38 patients (86.84%). They conclude that these techniques reduce the incidence of fracture stages, delay disease stage I and II progression and decrease hip pain and joint symptoms.

A publication by Hernigou *et al.* conducted a prospective evaluation of core decompression combined with the injection of concentrated autologous bone marrow cells in 116 patients (a total of 189 hips) (122). At least 5 years of follow-were part of including criteria; arthroplasty was required in only 6% of Steinberg stage I and II hips compared 57% of hips that were in stages III and IV.

Core decompression with vascularized grafting

Supplementation with vascularized grafts using fibula or iliac crest grafts, offers the potential advantage re-vascularization and promotion of osteogenesis of the femoral head. It also serves as a viable, strong support for the subchondral bone (Figure 6). Plakseychuk *et al.* compared vascularized to nonvascularized fibula grafting and demonstrated that vascularized fibular graft (VFG) significantly improved survival (86%) of pre-collapsed femoral heads after 7 years of surgery compared to nonvascularized grafting (30%) (125).

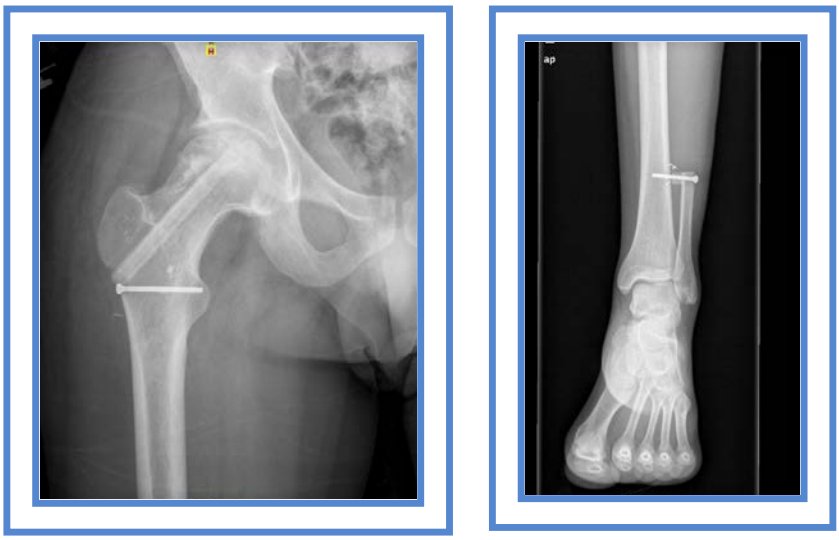


Figure 6: Young patient with ONFH of right hip treated by fibular autograft.

Yoo *et al.* were able to determine the long-term outcomes of VFG; with a mean follow-up of 13.9 years, they reported that 11% had failed and had undergone THA. Ficat-Arlet stages I and II hips showed no significant differences and hip survival was strongly associated with the age of the patient and lesion characteristics (size, location) (126).

Vascularized bone grafting has some downfalls, as it requires skillful microsurgery expertise and donor site morbidity, including motor weakness, sensory abnormalities, etc. has been reported in approximately 20% of the patients. This technique should be considered for patients without signs of collapse of the femoral head.

Osteotomies

Osteotomies aim to prevent femoral head collapse by shifting the osteonecrotic area from the weight-bearing zone of the hip joint, thus reducing

mechanical stress on the affected area. There are two broad types of osteotomies: angular intertrochanteric (varus or valgus) and rotational transtrochanteric (anterior or posterior). Successful osteotomy procedures greatly depend on the size of the lesion and careful assessment of extent of bone donor area to ensure that enough healthy tissue is available to be transferred to the bearing area of the acetabulum. A long-term outcome study published by Morita et al included 111 hips in 95 patients with a median follow up of 18,2 years showed a survival rate of 59%. They concluded that the 15-year outcomes after TRO for ONFH are unfavorable because osteoarthritic changes occur after five years post-operatively. (127)

Without a doubt, rotational osteotomies played an important role in the management of selected patients, but they can be difficult to perform and have a high risk of nonunion and poor long-term outcomes. Total arthroplasties in patients with previous osteotomies are often more difficult to manage than patients with ONFH who never had one. Intraoperative complications of osteotomy procedures include increased operative time, increased blood loss, high infection rates and poor long term outcomes in association with hip replacements after osteotomy (128).

Joint Arthroplasty:

- *Total joint replacement.*

Once the femoral head has collapsed or acetabular involvement has occurred, reconstructive surgery is the treatment of choice. Total hip replacement (Figure 7) is the most reliable treatment for achieving pain relief and prompt functional

convalescence in a single procedure, particularly in patients with signs of degenerative changes of the hip and joint.

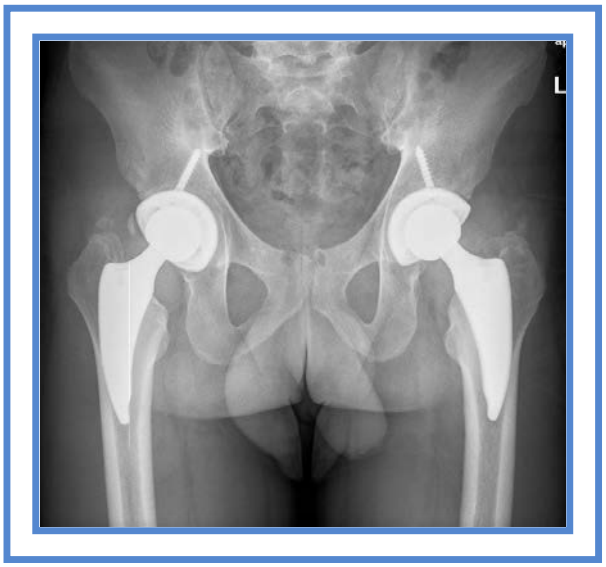


Figure 7: Bilateral Total Hip Arthroplasty.

The 2017 Australian Joint Register reported an 11.1 % of revision surgery in patients with total hip replacement with 16 years of follow-up with diagnosis of femoral head osteonecrosis. They also reported that patients with Osteoarthritis have a lower rate of revision (8.8%) compared with femoral head osteonecrosis, femoral neck fracture and dysplasia. Despite the overall good long term results of the hip replacement, patients with diagnosis of ONFH are usually young and very active patients and may require more than one surgery during their lifetime, for that reason hip resurfacing is an alternative surgical treatment.

Hip resurfacing arthroplasty

Hip arthroplasty for younger patients with ONFH represents a viable alternative for the patient with femoral head collapse when all other nonoperative strategies have been exhausted. Patients with ONFH are usually in their third to fifth

decade may have a life expectancy beyond the expected life span of the implant. Surgical alternatives for treating these patients should remain somewhat conservative.

Potential advantages of resurfacing over total hip replacement are lower dislocation rates, preservation of bone stock, and the ability to perform conversion to total hip arthroplasty if necessary.

Amstutz *et al.* published a long-term study of hip resurfacing in patients with femoral head osteonecrosis, they reported a 90,3% or survival rate (129). Revell *et al.* in their study suggested that the results of the hip resurfacing are better in male patients with good bone reserve while the results are not as promising in patients with osteonecrosis induced by corticoids or women with a narrow femoral neck (130).

7. - Experimental animal models of ONFH

Prior to testing potential protocols for effective ONFH treatment and to elucidate potential underlying mechanisms, animal models have been generated to mimic human disease. Criteria for such experimental models should include mature skeletal structures and that they be suitable for testing different possible etiologies of the disease, such as trauma, corticosteroid use, intravascular coagulation and alcoholism.

7.1. - Trauma-induced ONFH experimental models

Experimental models of ONFH resulting from trauma include three major groups: surgical vascular deprivation models, physical and chemical insult-induced traumatic ONFH models.

Surgical deprivation models

Nishino *et al.* established a dog model by provoking dislocation of the hip joint and ligation of the medial and lateral circumflex arteries and veins. At weeks 2 and 4, 80% of the animals exhibited widespread necrosis. The necrotic zones were detectable by MRI but only in early stages of ONFH partly attributed to a short follow-up time (131).

Hofstaetter *et al.* established an adult rabbit model of traumatic ONFH to test the effects of alendronate on bone repair processes (132). They surgically and completely removed the hip joint capsule, and cauterized circumferentially the periosteum and blood vessels covering the femoral neck to interrupt the blood supply to the femoral head. The ligamentun teres was also ligated. A 3-mm drill hole was made through the posterior femoral neck into the marrow cavity, which was then cauterized to interrupt the intramedullary blood supply to the femoral head. After surgery, rabbits were allowed full weight bearing and contralateral hips were used as control. They evaluated the femoral head using micro-computerized tomography (micro-CT) and histology and alleged that at 6 months after surgery, no collapse was seen, while at 12 months 2 out of 15 (13.3%) of the rabbits presented collapse of the femoral head. Some authors allege that one year might be too long for assessment of ONFH as it can be reversible, especially in quadrupedal animals (133). Observation

within a 6-month period might be also too long to show both temporal and spatial changes of ONFH and its collapse in spite of good evidence of advanced disease stages reported in this model study (134).

Many researchers have also reported surgical models of ONFH in rats (135,136) which was achieved by temporary dislocation of the femoral head during surgery and cutting the ligamentum teres. The periosteum at the base of the neck of the femoral head was incised together with the reflected capsular fibers twice by circumferentially sweeping the edge of the knife at a 1-mm interval around the bone. Levin et al observed the presence of ONFH even after 42 days of surgery and osteogenesis of the trabecular bone and no collapse of the femoral head (135). Rat models are to be regarded as good models for early-stage LCPD given the fact that these animals' growth plates remain unclosed and thus keep growing throughout their lifetime. Contrary to the study previously discussed, Peled *et al.* found distortion of the femoral head but no histologic findings of necrotic trabecular bone 42 days after surgery (136). Such structural changes could be explained by the surgery that traumatized small rat hip so as the subsequent instability of hip joint that might result in long-term joint dislocation.

Physical and chemical insult-induced ONFH models

Other physical insults, such as cryogenic and thermal insults, have been used for the induction of ONFH. Despite not being naturally occurring, its use has been widely implemented for mimicking ONFH as bone repair follows a practically similar path as those that follow naturally occurring ONFH (137).

In cryosurgery, the freezing process causes direct cellular and vascular injury. On the other hand, treatment with hyperthermia (between 43 and 45°C), raises the tissue temperature mildly for a certain period of time, which is expected to induce cell death by affecting membrane fluidity, cytoskeleton, protein and nuclear structure, and disruption of DNA replication (138).

A canine model of ONFH achieved by deep freezing processes and stripping of the soft tissue attachments from the femoral neck and intertrochanteric area was proposed by Malizos *et al.* (139). The induced ONFH from this model induced bone reparation to completion with no collapse of the femoral head. As mentioned earlier, although it does not fully simulate human cases of NOFH, it establishes a good model for further experimentation with cryosurgery-induced ONFH.

Manggold *et al.* established an ONFH sheep model induced by direct intraosseous injection of pure ethanol. After 12 weeks, all animals presented partial necrosis with conservation of joint cartilage and macro-circulation. One out of 10 of the animals died during the injection due to massive pulmonary embolism. Ethanol was chosen as the insulting agent as its toxicity is concentration-dependent and its enzymatic degradation complete. These sheep displayed high incidence of ONFH but mostly for early stages of disease progression (140).

Velez *et al.* developed a new surgically induced animal model of femoral head osteonecrosis, using a combined method with intracephalic nitrogen liquid injection with a cryoprobe and vascular ligation in 10 sheep. After 6 and 12 weeks

they confirmed the femoral head osteonecrosis lesion with MRI and histological analysis (141).

A novel animal model of ONFH induced using a magnetic resonance imaging-guided argon-helium cryotherapy system was established by Wang *et al.* (142). They induced the ONFH in 48 rabits divided into two different groups: Two vs one cycles of argon-helium cryotherapy and compared the histological and MRI changes at 4, 8 and 12 weeks. The percentage of empty lacunae in group I was higher than that in group II at weeks 4, 8 and 12 following surgery. In addition, a statistically significant difference was observed in the femoral head collapse rates between the two groups.

7.2. - Experimental models of ONFH with non-traumatic agents

Non-traumatic causes of ONFH can be broadly categorized into spontaneous, steroid-induced, lipopolysaccharide-induced, alcohol-induced and dysbaric osteonecrosis, with any given combination of several of these factors.

Spontaneous ONFH models

Spontaneously hypertensive rats have been known to develop ONFH suggested that this was attributable to abnormal epiphyseal cartilage and metaphyseal growth plate and subsequent ossification of these structures (143,144). Mihara *et al.* observed that when these rats were being fed in such a manner that they had to stand on their hind limbs, a higher incidence of ONFH was manifest. Weight-bearing was regarded as the cause of higher ONFH incidence (145).

Corticosteroid-induced models of ONFH

Corticosteroid treatment is a decisive risk factor for developing ONFH and in some instances, may even be the etiologic cause of osteonecrosis (8). Many researchers to induce ONFH have used administration of intramuscular methylprednisolone in rabbits.

A single administration of 20 mg/kg of intramuscular methylprednisolone (MPS) on rabbits displayed histological incidence of ONFH in proximal metaphyses 3 weeks after having received the steroid (146).

Using the same model, Kuribayashi *et al.* reported an incidence of 70% of histologically-evident osteonecrosis at the distal one-third and proximal one-third of the femur after 4 weeks of MPS administration (147). While Takao *et al.* only found bone marrow necrosis at the proximal metaphysis or diaphysis but no evidence of ONFH despite that continuous monitoring was observed (1, 3, 6 and 9 weeks post-induction) (148). Some authors might argue that this rabbit model is not a reproducible one and does not constitute a good model for emulating ONFH (149).

In 2009, Yang *et al.* established, for the first time, a model of steroid-induced ONFH in mice (150). Treatment consisted of orally administered dexamethasone for 12 weeks, either by continuous or intermittent administration. The latter displayed lower trends of ONFH (8%) when compared to animals receiving continuous administration of the steroid drug (45%). Histological evidence of osteonecrosis, however, was only identifiable in distal femora and at early stages that results in lower incidence of ON.

Lipopolysaccharide-induced models of OFNH

In 2001, Irisa *et al.* developed a rabbit model non-steroid and nontraumatic osteonecrosis by means of a single- and low-dose lipopolysaccharide (LPS) intravenous injection (10 μg/kg) (151). They describe it as a simple and reproducible model for the frequent development of multifocal and widespread osteonecrosis lesions. These studies showed an incidence of ONFH in 77% of treated rabbits after 4 weeks of the injection; 4/35 rabbits died after administration of LPS. Histopathological findings included organized thrombi in the intraosseous small-sized arteries and arterioles. Despite the fact that osteonecrosis was seen in 9% of the rabbits, only early-stages of ON were induced. They concluded that their model would be useful for elucidating the pathogenesis of non-steroid osteonecrosis in humans especially inflammatory hypercoagulability-induced as well as for developing preventive and therapeutic strategies.

Immune reaction-induced models of OFNH

Tsuji *et al.* induced serum sickness-associated ONFH in rabbits by administering 10 ml/kg of sterile heat–inactivated horse serum twice over the course of a 3-week period (152). This induced immune complex deposition in a similar fashion as SLE does in humans. Incidence of osteonecrosis suffered time-related changes: at 72 hours, no osteonecrosis was evident; at 1 week, there was an 86% incidence but after 3 weeks, the incidence decreased to 63%. Only early-stage osteonecrosis was observed and no cases of ONFH were documented.

Alcohol-induced models of ONFH

In an attempt to elucidate the underlying pathophysiology of alcohol-induced ONFH, Wang *et al.* tested a protocol for such induction in mice by intragastrical administration of spirits (20 ml/kg) containing 46% of ethanol (153). After 6 months, osteonecrosis was observed in the subchondral region of the femoral head. This model proves useful for the elucidation of the pathogenesis of alcohol-related ONFH in humans as well as for evaluating different therapeutic protocols.

Dysbaric-induced animal models of ONFH

In the 1980s, a dysbaric model of osteonecrosis in C57BL/6J mice was described by Chryssanthou et al (154). Animals were subjected to 75 psi (516.75 kpa) air pressure for a period of 2 to 3 hours followed by stage decompression. After a period of 2 months, histological evidence of osteonecrosis similar to those seen in human dysbaric-induced ONFH.

More than a decade later, a model a of dysbaric-induced osteonecrosis was performed using adult sheep exposed for a period of 24 hours to compressed air (2.6-2.9 atm absolute) and repeated 12 to 13 times during a 2-month period. All exposed sheep had decompression sickness and extensive bone and marrow necrosis in their long bones. According to the authors, radiographic analysis of these progressive lesions showed mottled to distinct medullary opacities and endosteal thickening characteristic of dysbaric osteonecrosis. Proliferating endosteal new bone, fatty marrow calcification, and appositional new bone formation were widely seen. This animal model seems like a suitable candidate for the investigation of pathogenesis, diagnosis, and treatment of dysbaric osteonecrosis.

8. - Biological treatments of ONFH

8.1. - Stem cell therapy

Bone marrow-derived stem cells.

Hernigou *et al.* proposed bone marrow cells implantation combined with core decompression for ONFH in 2000's (122). They pioneered the clinical application of cell-based strategies for the treatment of ONFH, by percutaneous injection of an autologous bone marrow concentrate into the necrotic area of femoral heads.

Such strategy is driven by the hypothesis that bone marrow cells can replace the exhausted trabecular bone structure and subsequently revive and remodel the necrotic bone. For this technique, a bone marrow aspirate is harvested from the iliac crest and the mononuclear cell fraction is isolated. After core decompression, the cell suspension is injected into the necrotic area (122,155).

Later studies, have also reported on their experience with stem cell therapy for osteonecrosis (7,156,157). Ganji *et al.* in a small series examined disease progression in patients who received core decompression alone versus those who underwent core decompression combined with stem cell injection (7). This was consistent with the MRI findings from more recent studies that also showed that the disease did not progress in patients who received core decompression combined with stem cell injection versus those who received core decompression alone (158). Moreover, three patients in the stem cell injection group exhibited marked improvement in their symptoms as well as imaging findings. On the other hand, ten patients in the core decompression-alone group experienced deterioration of their

symptomatology and MRI findings. Of these patients, three required subsequent conversion to total hip arthroplasty. Remarkably, two patients with ARCO stage 3 who received stem cell injection also noted a marked improvement in their condition and the MRI appearance (158).

In the same study, patients reported marked improvement of functional status of as measured by Western Ontario and McMaster University Osteoarthritis Index (WOMAC) questionnaire and the visual analogue scale (VAS) pain index. The mean WOMAC and VAS scores in all patients improved significantly with the most marked improvement being in the group of patients that received bone marrow transplant (158).

More recent work by Gangji *et al.* was reported in a prospective, double-blinded trial conducted on 19 patients (24 hip joints) with a 5-year follow-up, who received either core decompression alone or core decompression with supplementation of bone marrow concentrate (123). The results showed that eight out of 11 hip joints in the core decompression group had disease progression with structural breakdown of the sub-chondral bone and only three out of 13 joints shared this feature in the bone marrow concentrate group. Despite these encouraging results, the clinical value of these studies is limited because of short-term follow-up periods and low number of patients.

Cultured bone marrow stem cells

The *ex vivo* expansion and later administration of MSCs, in contrast to bone marrow cell concentrates, are controlled by regulatory authorities, such as the US Food and Drug Administration (FDA), among others. In general, the selection of

MSCs is achieved by cell adherence to tissue culture plastic after phase gradient separation with Ficoll or Percoll.

This experimental approach offers the therapeutic potential for the treatment of cortico-steroid-induced ONFH and was studied by Müller *et al.* in 2008 (159). They harvested bone marrow aspirates from the posterior iliac of five patients and crest and MSCs were isolated and expanded for three passages. The MSCs were resuspended in 0.1% serum albumin-supplemented saline and transplanted into the necrotic area. After follow-up period of 16 months, none of the patients showed signs of disease progression. As there was no control group, the effect cannot be easily attributed to the injected cells.

Other methods for cell delivery other than suspension have been described by Nöth *et al.* (160), such as ceramics, collagen sponges, hydrogels, and biodegradable polymers. These scaffolding agents can easily be delivered by core decompression into the necrotic area and as new tissue forms, they undergo degradation processes as they are biocompatible and excretable. It has also been suggested for the treatment of ONFH, specific autologous or allogenic bone grafts as well as synthetic materials like beta-tricalcium phosphate (β-TCP) as carriers for these strategies (3,160).

Kawate *et al.* reported a clinical study of three patients with advanced stages of cortisone-induced osteonecrosis (Steinberg stage III or IV) treated with a vascularized fibular graft combined with a synthetic β-TCP ceramic and bone marrow-derived MSCs (161). One month before core decompression, 15 mL of bone marrow aspirate was taken from the iliac crest for MSC isolation and expansion in autologous serum. Then, after 10 days, MSCs were seeded onto the β-TCP granula and cultured for 2 more weeks. Once core decompression had been performed, the

defect was filled with the MSC-seeded β-TCP granula and transplantation of a vascularized fibular graft was made. Promisingly, during the 34-month follow-up, patients did not exhibit disease progression although no control group was used and only a small number of patients were recruited for the study.

Similar work by other authors also treated ONFH with autologous stromal cell-seeded β-TCP granules (162). All patients had been diagnosed with early ONFH (ARCO stage II). Briefly, bone marrow aspirates (100 mL) were harvested from bilateral posterior iliac crests. The aspirates were expanded for 12 days in autologous serum-supplemented medium. After surgical exposure of the proximal femur, a bone plug was removed and treatment of small AVN lesions (ARCO stage IIA) was performed with a K-wire placed centrally into the necrotic area. For the treatment of more extensive lesions (ARCO stage IIB and IIC), two drill holes were used. Subsequently, β-TCP granula was seeded with the suspension. This group of researchers has treated 4 patients using the described technique without signs of disease progression at a 24-month follow-up (163).

Allogenic bone marrow-derived stem cells

The use of allogenic instead of autologous MSCs for the treatment of ONFH would seem like treatment panacea due to its economic and logistic advantages. However, allogenic MSCs harbor the danger of disease transmission and immunological rejection just as is seen in organ transplant rejections. Given that no other available treatments for more severe disorders such as osteogenesis imperfecta, allogenic MSC transplantation appears to be more suited for such conditions (164).

In a Recent study by Gao *et al.* (165) the implantation of a novel nanoscaled core decompression rod combined with umbilical cord mesenchymal stem cells was used. They aimed to cure 12 cases of early ONFH and offer complete follow-up for all of them. Their mean period of follow-up was 12 months. They treated early osteonecrosis of the femoral head by organically combining two main elements of bone tissue engineering, cells (umbilical cord mesenchymal stem cells) and scaffolds (nanoscaled core decompression rods). They found significant clinical effects with an increase of the average grade of hip joint according to the standard Harris scoring.

8.2. - Intra-arterial injection of stem cells

A study by Mao *et al.* (166) were performed in order to determine the benefits of combination treatment with mechanical support and targeted intra-arterial infusion of peripheral blood stem cells (PBSCs). These cells were mobilized by granulocyte-colony stimulating factor (G-CSF) via the medial circumflex femoral artery on the progression of osteonecrosis of the femoral head (ONFH). They studied a total of fifty-five patients (89 hips joints) with early and intermediate stage ONFH to whom they randomly assigned either combination treatment or mechanical support treatment, which served as the control group. All affected hips received mechanical support treatment (porous tantalum rod implantation). Afterwards, hips in the combination treatment group received the targeted intra-arterial infusion of PBSCs. At each follow-up, Harris hip score (HHS) and Association Research Circulation Osseous (ARCO) classification were used to evaluate the symptoms and progression of osteonecrosis. Total hip arthroplasty (THA) was assessed as an endpoint at each follow-up. At 36 months, 9 of the 41 hips (21.95%) in the control group progressed to clinical failure and underwent THA whereas only 3 of the 48 hips (6.25%) in the

combination treatment group required THA ($p = 0.031$). This clinical trial confirmed that the combination treatment might be a safe and feasible choice for the treatment of early or intermediate stages of ONFH.

In 2016, Chen *et al.* (167) drove a retrospective analysis of the clinical effects of transplant of mesenchymal stem cells (MSCs) derived from human umbilical cord-derived MSCs (hUCMSCs) for the treatment of osteonecrosis of the femoral head (ONFH). Nine patients categorized as ARCO stages II - IIIA were enrolled in the and hUCMSCs were grafted by intraarterial infusion. MRI findings revealed that at 12 and 24 months after treatment, the necrotic volume of the femoral heads was significantly reduced, and no obvious abnormalities were observed. The data produced by these authors seems to indicate that intra‐arterially infused hUCMSCs migrate into the necrotic field of femoral heads and differentiate into osteoblasts, thus improving the necrosis of femoral heads. This finding suggested that intra‐arterial infusion of hUCMSCs MSCs is a reasonable and quite safe method for the treatment of femoral head necrosis.

A 5-year follow-up study by Mao *et al.* (168) consisted in investigating the efficacy and safety of targeted delivery of autologous bone marrow mononuclear cells (BMMCs) via medial circumflex femoral artery for the treatment of osteonecrosis of the femoral head (ONFH). The study included 62 patients (78 hips) with ONFH. All of these patients were treated with BMMCs perfusion via medial circumflex femoral artery. The concentrated BMMCs (30–60 ml) were gained from autologous bone marrow (100–200 ml) harvested from anterior iliac crest and then were intra-arterially perfused into the femoral head. Ficat stage was used to classify the radiological stage of ONFH. Harris hip score was used to evaluate the clinical

symptoms of osteonecrosis. Ficat stage and Harris hip scores were assessed at onset of treatment at 6, 12, 24, 36, 48 and 60 months after the initial treatment. Total hip arthroplasty (THA) was also assessed as an endpoint at each follow-up. After the five-year period, the authors were able to conclude that autologous BMMSC perfusion via the medial circumflex femoral artery can relieve symptoms, improve hip function and delay the progression of ONFH. The clinical outcome is better when it is applied prior to the collapse. This work demonstrates that autologous BMMSC perfusion via the medial circumflex femoral artery is a safe, effective and minimally invasive to treat early-stage ONFH.

8.3. - Bioactive molecules

Recent work by Sun *et al.* (169) aimed to compare the clinical outcomes of impacted bone graft with or without recombinant human bone morphogenetic protein-2 (rhBMP-2) for osteonecrosis of the femoral head (ONFH). They examined the effect of bone grafting through a window at the femoral head-neck junction, known as the “light bulb” approach. A combination of artificial bone mixed with or without rhBMP-2 was delivered. A total of 42 patients (72 hip joints) were followed-up for an average of 6.1 years. The clinical effectiveness was evaluated by Harris hip score (HHS). The radiographic follow-up was evaluated by pre-and postoperative X-ray and CT scan. Excellent, good, and fair functions were obtained in 36, 12, and 7 hips, respectively. The group with MBP showed a survival rate of 81.8% and the group without MBP, 71.8%. However, the survival rate was 90.3% in ARCO stage IIb and IIc, and only 34.6% in ARCO stage IIIa ($P<0.05$). They concluded that good and excellent mid-term follow-up could be achieved in selected patients with ONFH

treated with impacted bone graft operation and that the rhBMP-2 might improve the clinical efficacy and quality of bone repair.

In 2016, Gao *et al.* (170) evaluated the effectiveness of core decompression combined with implantation of bone marrow–derived cells (BMDC) and rhBMP-2 for osteonecrosis of femoral head (ONFH) after femoral neck fractures in children and adolescents. The study included 51 patients, aged 11.4–18.1 years, with ARCO stages I–III ONFH after femoral neck fractures. The hips were divided into two groups based on whether the lateral pillar of the femoral head (LPFH) was preserved: LPFH and non-LPFH groups. All patients were followed up clinically and radiographically for a minimum of 5 years. The results showed that 86.3% of the patients had improved clinical outcomes. Radiologically, 17.6% exhibited collapse onset or progression of the femoral head or narrowing of the hip joint space, and one patient in the non-LPFH group required hip arthroplasty due to worsening of the condition. They conclude that the technique provides an effective therapeutic option for children and adolescents with ONFH following femoral neck fractures. The report it relieves hip pain and prevents the progression of osteonecrosis in young patients lasting more than 5 years after surgery.

A retrospective evaluation was performed by Lieberman *et al.* (3) on 15 patients (17 hip joints) with symptomatic osteonecrosis of the hip treated with core decompression combined with an allogeneic, antigen-extracted, autolyzed fibula allograft and 50 mg of partially purified human bone morphogenetic protein and non-collagenous proteins. The average duration of clinical follow-up of the patients ranged between 26 and 94 months). The osteonecrotic involvement of the hip was classified by plain radiographs using a modification of the Ficat staging system and

MRI evaluations. Fifteen hips were classified as Ficat Stage IIA, one hip from one patient was classified as Ficat Stage IIB, and one hip from another patient was classified as Ficat Stage III. The procedures were a clinical success in 93% of hips with Stage IIA disease. Three out of 17 hips had radiographic progression (Ficat Stages IIA, IIB, and III) of the femoral head and were converted to total hip replacements.

Studies by Kuroda *et al.* (171) evaluated the safety and clinical outcomes of a single local administration of gelatin hydrogel impregnated with recombinant human fibroblast growth factor (rhFGF)-2 for the treatment of the precollapse stage of osteonecrosis of the femoral head (ONFH). For evaluation, 10 patients with ONFH received a single local administration of 800 μg of rhFGF-2-impregnated gelatin hydrogel and were followed up for one year. The surgery was performed using a minimally invasive technique involving a 1-cm skin incision, and walking was allowed from day one postoperatively. Five of the patients experienced various adverse events, including one complication from spinal anesthesia. Nevertheless, patients completely recovered from these events. The mean clinical scores significantly improved by one year postoperatively compared with the pre-operative scores (before vs. after: visual analog score for pain, 21.2 vs. 5.3 mm; UCLA activity score, 5.5 vs. 6.6; Harris hip score, 81.0 vs. 96.9 points). There was only one case of femoral head collapse; however, this occurred in a hip with extensive necrosis. Stage progression and collapse did not occur in the other nine cases. Computed tomography confirmed bone regeneration in the femoral heads. They conclude that clinical application of rhFGF-2-impregnated gelatin hydrogel for patients with precollapse ONFH was feasible and safe.

8.4. - Platelet-rich plasma

Recent work by Samy *et al.* (172) described early results of treatment of ONFH by replacement of the necrotic segment after multiple drilling with bone graft and PRP covered by collagen sheet to augment healing process. They performed a prospective study of 40 hips in 30 patients with a mean age 36.7 years. The indication for the operation was restricted primarily to modified Ficat stages IIb and III. Out of all the patients, 40% of the hips had stage IIb and 60% had stage III ONFH. The mean period of follow-up was 41.4 months. All patients were assessed clinically during the pre- and post-operative period according to the Harris Hip Score (HHS), Visual Analog Score (VAS) and radiologically by X-rays. Moreover, magnetic resonance imaging (MRI) was done preoperatively to confirm the diagnosis and every 6 months postoperatively for assessment of healing. The operative procedure included removal of necrotic area with drilling and posterior cavity filling with a composite of bone graft mixed with platelet-rich plasma. The mean HHS improved from 46.0±7.8 preoperatively to 90.28±19 at the end of follow-up (P<0.0001). The mean values of visual analog score were 78±21 and 35±19 at preoperatively period and final follow-up, respectively. They found that the use of PRP with collagen sheet can increase the reparable capacity after drilling of necrotic segment in stage IIb and III ONFH.

Other methods of delivering platelet-rich plasma apart from bone grafting have also been described. Martin *et al.* (173) described a surgical procedure for the treatment of osteonecrosis of the femoral head using a minimally invasive technique. The procedure was limited to patients with pre-collapse osteonecrosis of the femoral head (Ficat Stage I or II). Following the decompression of the femoral head, adult

mesenchymal stem cells obtained from the iliac crest and platelet rich plasma are injected into the area of osteonecrosis. Patients were then discharged from the hospital using crutches to assist with ambulation. This novel technique was used on 77 hips. Twenty-one per cent of these hips progressed to further stages of osteonecrosis, ultimately requiring total hip replacement. Significant pain relief was reported in 86% of patients (n= 60), while the rest of patients reported little or no pain relief. There were no significant complications in any of the patients. We found that the use of a minimally invasive decompression augmented with concentrated bone marrow and platelet rich plasma resulted in significant pain relief and halted the progression of disease in a majority of patients.

HYPOTHESIS AND OBJECTIVES

9. -Hypothesis

The use of a calcium phosphate foam loaded with bone marrow concentrate or BMP-2 associated to core decompression stimulates greater bone regeneration compared to core decompression alone in a new experimental model of osteonecrosis of the femoral head in a sheep preclinical model.

10. - Objectives

1- Establish a new modified experimental model of pre-collapse ONFH in sheep by using percutaneous cryotherapy with helium-argon gases.

2- Verify the ability of a calcium phosphate foam loaded with BMP-2 or bone marrow concentrate associated with a core decompression to stimulate bone regeneration in an animal model in sheep of ONFH.

3- Compare the isolated core decompression with the implantation of a calcium phosphate foam loaded with BMP-2 or bone marrow concentrate associated with a core decompression using MicroCT Scan, biomechanical and histological evaluation.

4- Evaluate the safety of the use of β-TCP in combination with bone marrow concentrate or BMP-2 as a treatment of ONFH in an animal model in sheep.

MATERIAL AND METHODS

The Ethical Committee of Animal Experimentation (CEEA) of the Institut de Recerca del Hospital Universitari Vall d'Hebron (VHIR) approved the experimental protocol for this project with registration number 72/13 CEEA.

Animal care and experimental procedures were carried out in accordance with the European Directive 2010/63 / EU, the national legislation in force at any time (Real Decreto 53/2013), and local legislation (Decret 214/1997), which establishes the basic standards applicable for the protection of animals used for experimentation and other scientific purposes, including teaching.

11- Material

11.1 Animal

We used 20 Adult Ripollesa x Lacunae´s Race female Sheep (Figure 8) in our study. These animals were transferred from the distributor (A.M Animalia Bianya S.L. Girona. Spain) in an authorized vehicle to the Vall d´ Hebron Research's institute (VHIR) animal facility one week before the procedure in order to acclimate them. An anteroposterior and lateral tibial X ray (Figure 9) were performed to ensure closure of the anterior tibial tuberosity growth plate, also a pelvic ultrasound were done to diagnose a possible pregnancy.

Figure 8. Ripollesa Sheep in the stable.

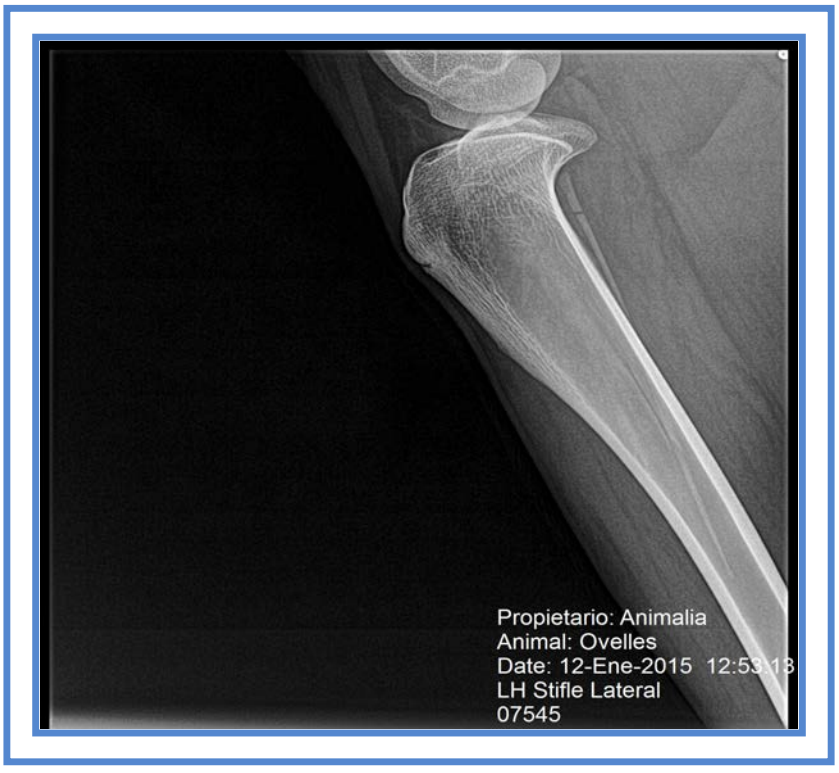


Figure 9. Lateral X Ray of the Tibia, showing closure of the anterior tibial tuberosity growth plate.

Animal facility´s environmental parameters were daily registered throughout the study. Temperature was stable with range of 17 to 21 Celsius degrees and humidity from 45-65%. The air driven was 100% external, pre-filtered and filtered with 95% efficiency and with a renewal rate of 15-20 cycles/hour. The photoperiod was programmed in 12 hours of light and 12 hours of darkness, being the light schedule of 8 to 20 hours.

All animals had free access to water dispensed by an automatic drinking fountain. The chemical and microbiological quality of the water was regularly monitored. The diet was composed of hay *ad libitum*, a granular feed supplement was administered twice daily and a block salt supplement, throughout the experimental period.

11.2 Osteonecrosis of the femoral head (ONFH) induction material

ONFH Induction System:

A percutaneous, non invasive femoral head osteonecrosis model was developed using the Cryo-44 Crioprobe, Endocare Cryocare, HealthTronics, consist of a compact easy to operate console (Figure 10) that delivers cold tempeatures to targeted tissue, vía connected Cryoprobes (Figure 11), the system utilizes inert Argon and Helium gas.

Figure 10. Cryotheraphy console and gas connections.

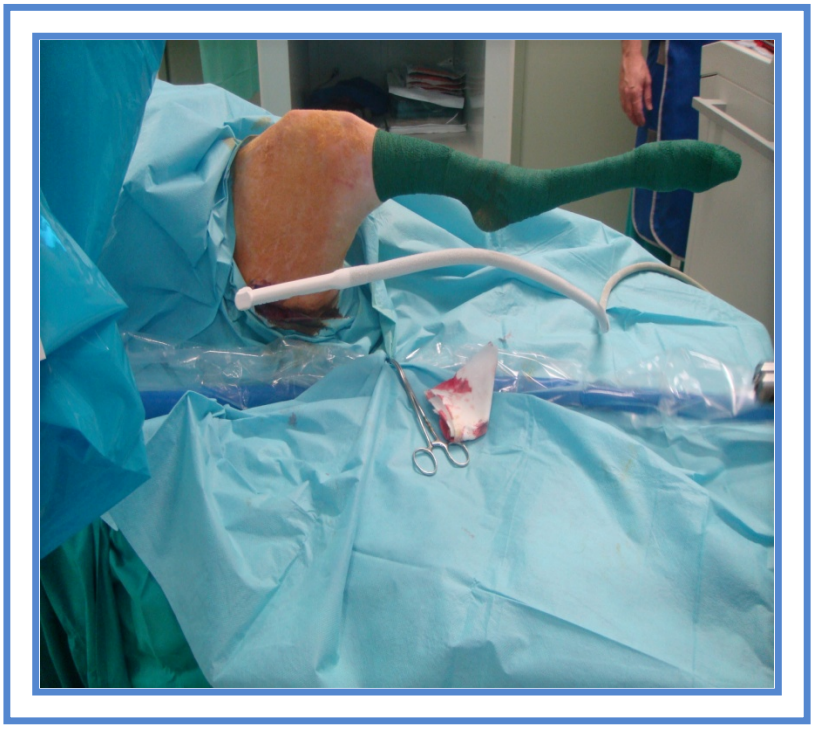


Figure 11: The cryoprobe inserted into the femoral head of the sheep.

Core Decompression instruments:

- Basic surgical instruments.
- 2,7 mm Kirschner Wire.
- Chuck with T-Handle.
- 5,0 mm Trephine.

Figure 12: A 5,0 mm Trephine.

Concentrated Bone marrow aspiration instrumental:

- T-Lok Bone Marrow Biopsy Needle 11ga x 4in, Argon medical devices)
- 5 Units of 10ml Syringes.
- Citrate dextrose ACD solution (Grifols).
- Dulbecco's phosphate-buffered saline DPBS (Sigma-Aldrich).
- Ficoll Paque density gradient.
- Heparin 5000 UI/ml.
- Centrifuge.

rhBMP2:

- Recombinant human bone morphogenetic protein-2 (rhBMP-2) TruScient^{r}, comercially avaliable form Pfizer.

12- Methods

12.1 Anesthetic protocol

All surgical procedures followed the anesthetic and surgical protocols established in the experimental operating rooms of the Vall d'Hebron Research Institute (VHIR) animal facility. The preoperative regimen was initiated thirty minutes before the surgery, Buprenorphine (0.02 mg / kg) and midazolam (0.5 mg / kg) mixed in the same syringe intramuscularly in the shed for sheep. Once sedated the sheep was transferred to the pre-weaning area, a venous access was obtained through the cephalic vein and the surgical area was prepared by performing a shaving and cleaning with iodized solution. From the pre-weaning, the sheep was moved to the operating room

(Figure 13) and positioned on the surgical table in supine position and the non-invasive blood pressure cuff, electrodes for electrocardiogram, rectal probe for body temperature, and pulse-oximetry sensor were applied Subsequently, an adequate anesthetic level with intravenous propofol (5 mg / kg) and endotracheal intubation was performed with an 11F tube connecting to a mechanical ventilator. The anesthetic level was then maintained with isoflurane, monitoring ventilatory pressure, volumes, inspired fraction of oxygen and the partial pressure of CO2. At the end of the procedure, the anesthetic was closed, reversing the anesthetic level until the sheep had spontaneous ventilation, and then extubated.

Post-operative care included analgesia, antibiotic prophylaxis, and surgical wound care. Prophylactic antibiotic with ceftriaxone 1 g intravenously was administered to the animal. The antibiotic regimen continued in the first postoperative week by the administration of long-acting amoxicillin (48h) 150 mg / kg in an alternate day intramuscular injection. When the animal was fully awake and active, it was transferred to the housing area.

Figure 13. Institut de Recerca del Hospital Universitari Vall d'Hebron (VHIR) Operating Room .

12.2 Femoral head osteonecrosis (FHO) induction technique:

With the animal anesthetized and in supine position, the entry point is located at the level of the lateral cortical of the proximal femur under radioscopic control by means of the anteroposterior and axial view (Figure 14). A 2.0 mm Kirschner wire is inserted through the femoral neck to define the entry point in the cortical and the exact location of the center of the femoral head. Subsequently, The wire is then removed and the Cryo-44 Crioprobe is inserted percutaneous using the same direction and orientation of the K wire (Figure 15).

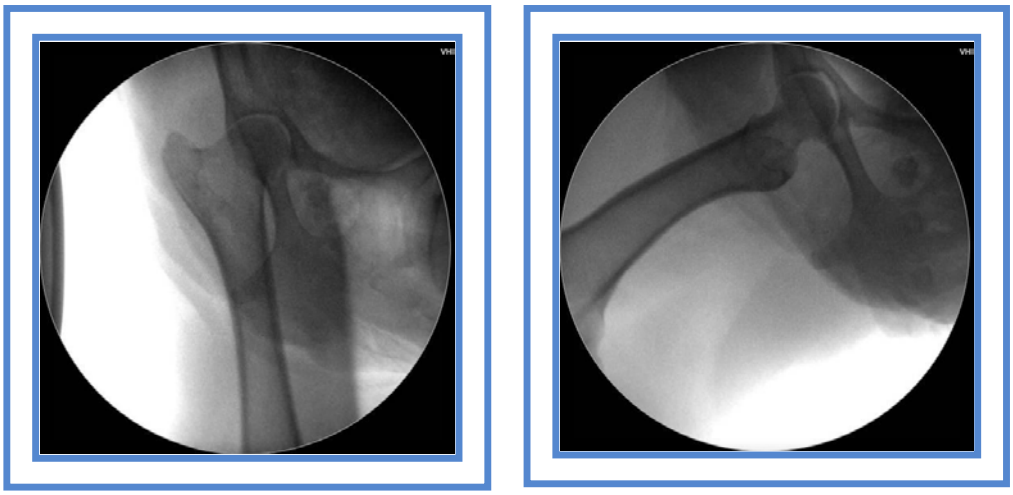


Figure 14. Antero-Posterior and axial X ray views of the proximal femur.

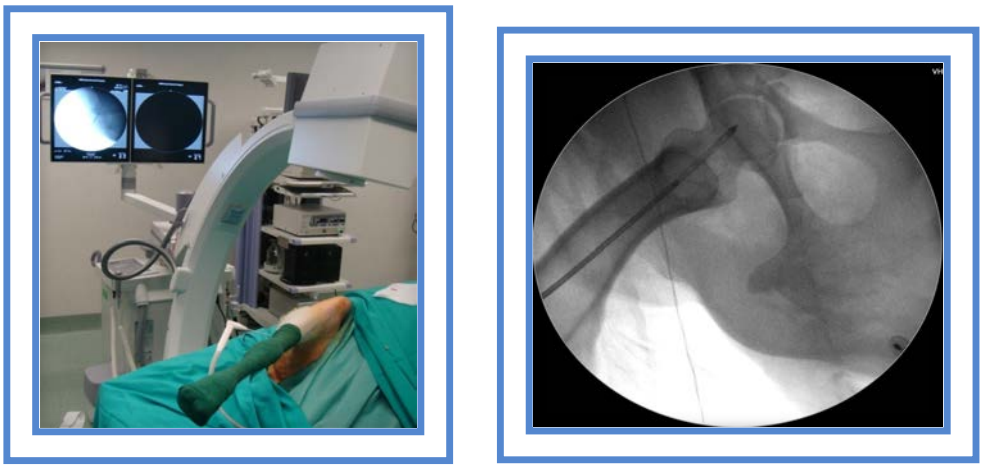


Figure 15. The cryprobe placed under fluoroscopy guidance into the femoral head.

Once the probe was placed at the point of induction of the lesion in the femoral head, the cryotherapy procedure was performed. Two consecutive cycles of cold (-130 ° C - 150 ° C) and heat (10 ° C - 20 ° C) induced by the argon and helium gases (Air Liquide), respectively, alternate for 10 minutes each (Figure 16). Once the cryotherapy cycles wew completed, the cryoprobe were removed and the wound was closed with Vycril® 2-0 sutures (Ethicon). When the animal was fully recovered from the anesthesia it was returned to the shed with the rest of the sheep.

Figure 16. Screen of the cryotherapy console showing the temperature and time achieved in ONFH induction surgery.

12.3 Core Decompression technique:

The core decompression was performed in all groups 6 weeks after the femoral head osteonecrosis induction surgery. The sheep was placed in supine position and the anesthetic protocol was initiated. A 2 mm Kirschner wire was inserted through the femoral neck into the femoral head as a guide. Subsequently, a biopsy trocar (5 mm external diameter and 2.5 mm internal diameter) was inserted parallel to the guide and following the same direction where the induction cryotherapy of the ONCF lesion was performed (Figure 17). With a hammer the trocar was inserted into the femoral head necrotic lesion under fluoroscopic guidance. Once the decompression was finished, the trocar was removed and the cylindrical necrotic bone was used in order to macroscopically confirm the femoral head osteonecrosis and also a histological analysis was performed.

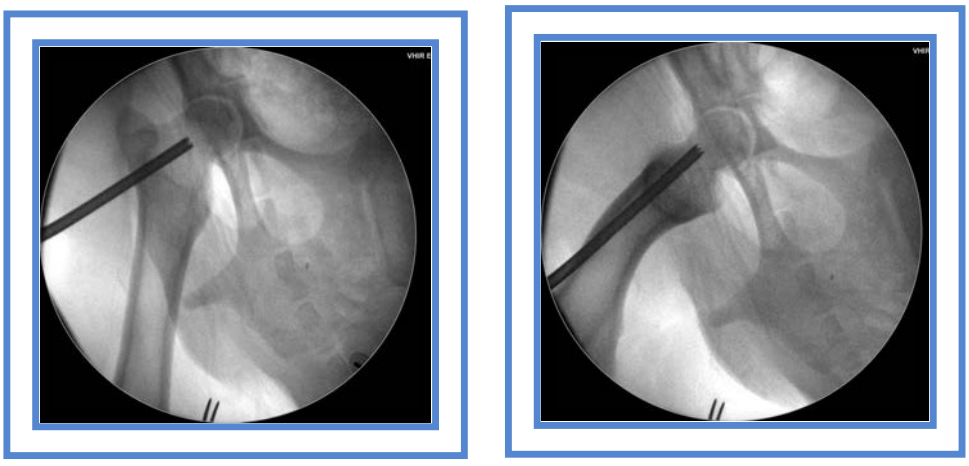


Figure 17. Antero-Posterior and axial X Ray views of the proximal femur with the 5mm trocar placed into the femoral head in the core decompression surgery.

Core Decompresion associated to Implantation of calcium phosphate granules (β-TCP) loaded with bone marrow concentrate:

Spherical beta-tricalcium phosphate (β-TCP) granules were produced from the sintering of calcium-deficient hydroxyapatite granules. The chemical composition of the material was confirmed by X-ray diffraction. Surface morphology was characterized by scanning electron microscopy; the surface area was quantified by nitrogen adsorption, and the porosity ay mercury porosimetry. The size distribution and sphericity of the granules were determined by image analysis, from optical microscopy and scanning electronics. In order to obtain a stable injectable composition the (β-TCP) granules were mixed with Sodium alginate (Figure 18). Six weeks after the femoral head osteonecrosis induction and following the anesthetics protocol, the animal is placed in supine position, the sternal zone is aseptically prepared. A puncture was made with a 11G Trocar in the anterior part of the sternum (Figure 19). Two mL of bone marrow were aspirated before each trocar redirect, until a total volume of 50 mL mixed with citrate dextrose anticoagulant solution (ACD, Sigma-Aldrich) was obtained. The bone marrow was diluted with DPBS solution (Sigma-Aldrich) and then centrifuged for 30 minutes in the Ficoll-Paque density gradient to isolate the mononuclear cell band. (Figure 20) The final volume of the mononuclear cell concentrate (0,5 ml) obtained was mixed with 500 mg of β-TCP granules, 0,6 ml of alginate and 0,3 ml of calcium chloride under sterile conditions. Simultaneously the core decompression technique was performed as mentioned above. Once the procedure was finished the mixture of bone marrow concentrate was introduced with the β-TCP granules through the trocar placed at the femoral head with the aid of a blunt punch (Figure 21).

Figure 18. Injectable composition of the β-TCP granules mixed with Sodium alginate.

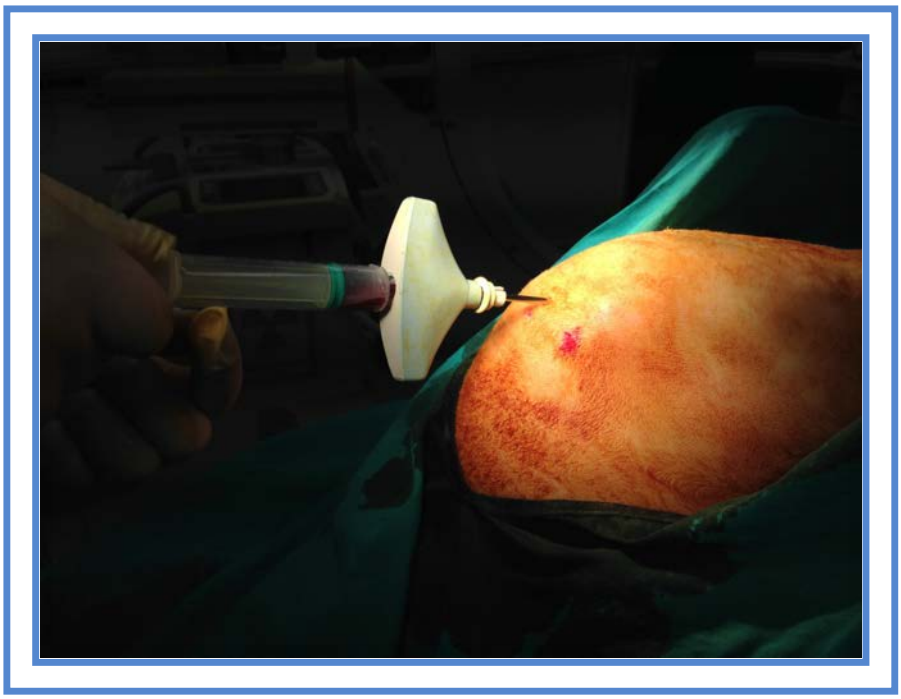


Figure 19. Bone Marrow extraction technique in the anterior part of the sternum of the sheep.

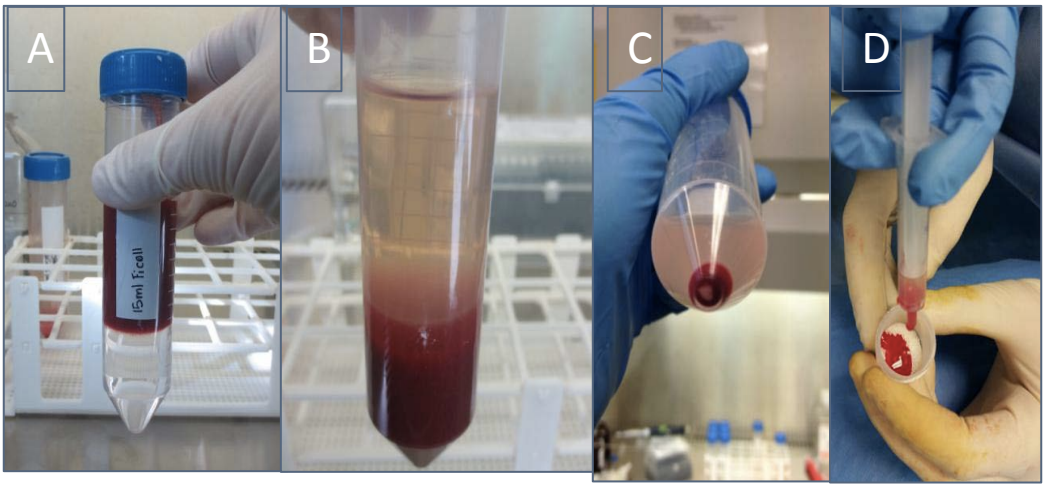


Figure 20. A. Bone marrow diluted in DPBS with Ficoll-Paque before centrifugation. B. Centrifugation of the bone marrow through the Ficoll-Paquell density gradient and obtaining the ring of mononuclear cells. C. Obtaining the mononuclear cell pellet. D. Mixture of the bone marrow mononuclear cell concentrate with the β-TCP granules.

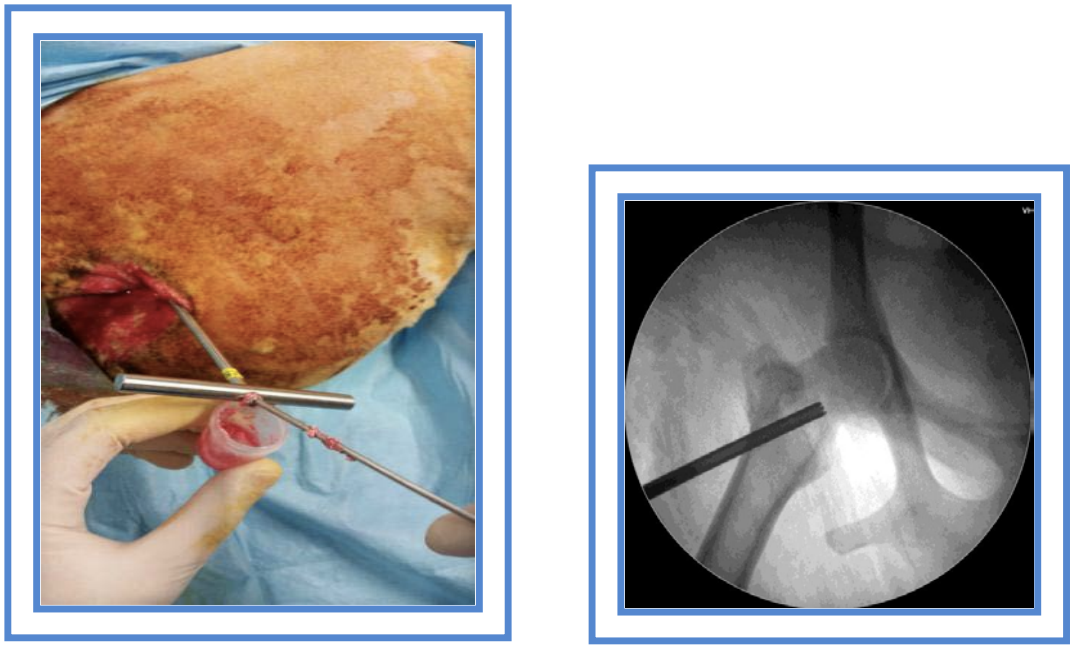


Figure 21. Implantation of the mixture of bone marrow concentrate with the β-TCP granules through the trocar. B. Radiological follow-up of the implantation in the ovine femoral head.

Core Decompression associated to Implantation of calcium phosphate granules (β-TCP) loaded with BMP-2:

Six weeks after the femoral head osteonecrosis induction and following the anesthetics protocol, the animal was placed in supine position. Following the Truscient commercial kit (Figure 22): a 6 mL syringe and needle was used, 3.2 mL of solvent was removed and slowly injected the solvent into the lyophilisate vial to achieve a 0.2 mg/mL solution of rhBMP-2, the final volume was then mixed with 500 mg of β-TCP granules, 0,6 ml of alginate and 0,3 ml of calcium chloride under sterile conditions. Core Decompression technique was performed as mentioned above and the granules loaded with rhBMP-2 were introduced through the trocar inserted into the femoral head (Figure 23)

Figure 22. rhBMP-2 commercial Kit.

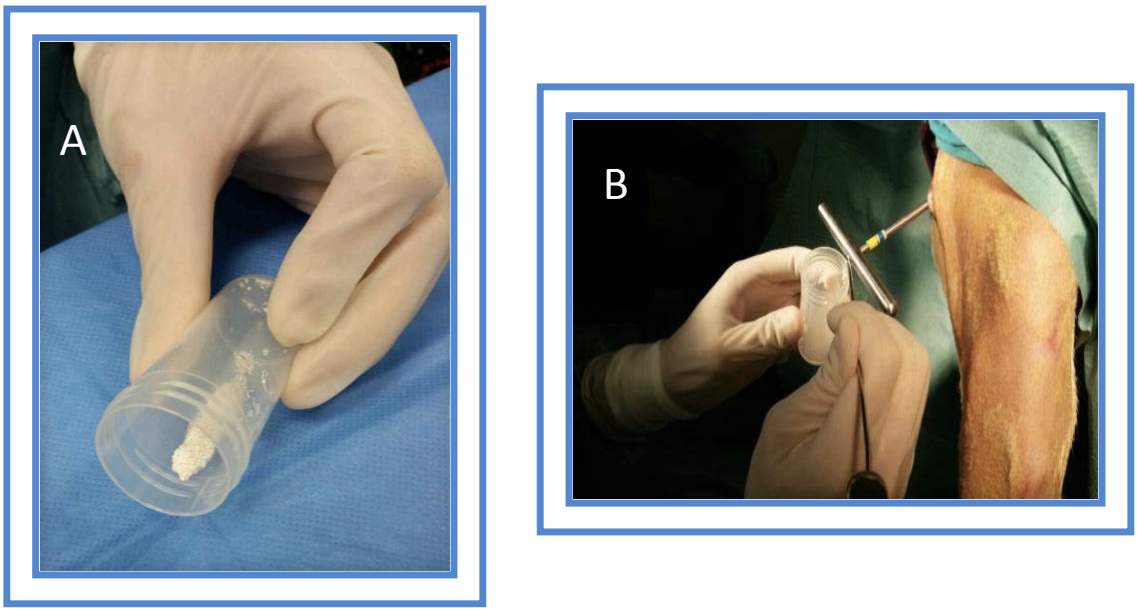


Figure 23. A. Appearance of the β-TCP granules with rhBMP-2 before its implantation. B. Implantation of β-TCP granules with rhBMP-2 through the trocar.

12.4 Clinical Evaluation

Sheep were monitored daily throughout the study period. Control includes: appearance and body condition of the sheep including weekly weight control, observation of its behavior and habits, food and drink intake, clinical follow-up of the procedure and any incidence. Caregivers, technicians and research personnel involved, animal welfare advisers and veterinarians who assisted the animals carried out the observation of the animals. During the 24 hours a remote surveillance system was also available through a webcam that allowed the sheep to be recorded and evaluated. In addition, it allowed being able to study its conduct without the presence of people (Figure 24).

Figure 24. Images from the remote surveillance camera showing the sheep in the animal facilities.

12.5 Euthanasia protocol

Six weeks after Core Decompresion and additional treatments the sheep was sacrificed. The animal was premedicated with intramuscular midazolam 0,5 mg/Kg. The sheep was transferred to the operating room and an endovenous catheter was placed and the right hip was shaved. A dose of 2-4 mg/ kg of Propofol and 3 g of Tiopental was administered. The death of the sheep was certified and the right femur was extracted using the material of sheep hip surgery. The soft tissue and surrounding muscles were resected from the femur. (An osteotomy cut was then performed distal to the minor trochanter (Figure 25).

An X ray and MicroCT scan evaluation was performed to the proximal femur. A cut into two halves using an Exakt 310 diamond band (Exakt, Germany) was performed. One of the cut halves was used for the biomechanical test and the other half was immersed immediately in formalin for subsequent histological analysis

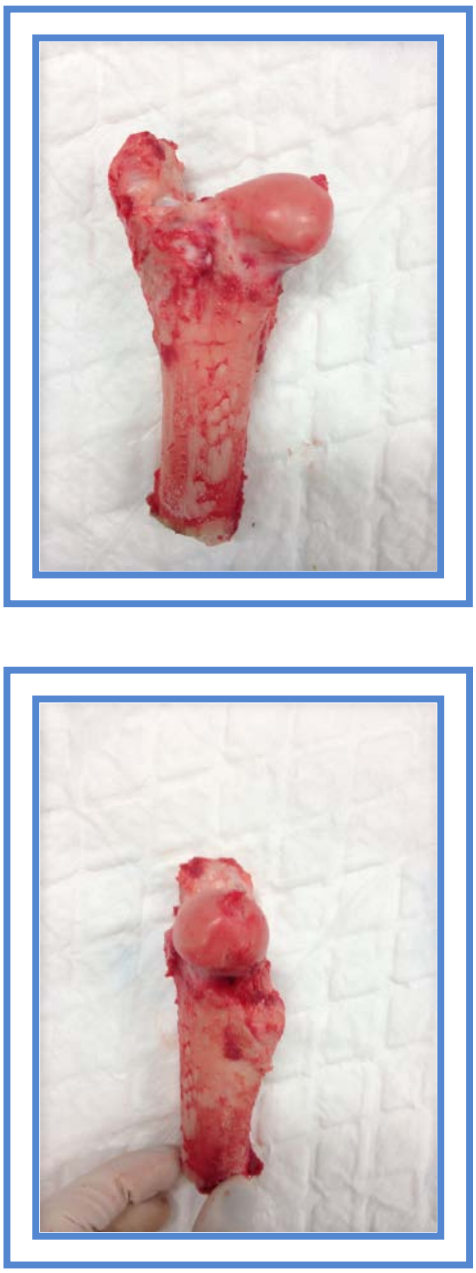


Figure 25. Anatomopathological piece of the right proximal femur

12.6 Micro CT Scan evaluations:

Once the femoral heads were removed from the animals that completed the study period, x-rays were taken on different views of the femoral heads with the fluoroscopy device (OEC Fluorostar 7900 Compact, GE Healthcare) and processed by microCT (Quantum GX microCT Imaging System , Perkin Elmer). The Characteristics of microCT were: FOV 40mm, 90 kv, 160uA, voxel size 0.000512mm3 (Figure 26).

Figure 26. Quantum GX micro CT Imaging System.

The AMIDE (Medical Imaging Data Examiner) software was used for the selection of regions of interest and their subsequent analysis. Obtaining VOI's (Volumes Of Interest) of 2mm^3, the choice of a smaller volume is intended to reduce the variability factor between samples due to the different orientations of the central decompression channels within each femoral head. In this way, it is possible to

minimize as much as possible those areas where there is no bone tissue and that would alter the values obtained increasing its error and its variability.

The VOIs are selected in random sections and are distributed in the following groups (Figure 27):

- VOI's control zone (trabecular tissue of the femoral head) n = 4
- VOI's zone adjacent to the cylinder (regeneration zone) n = 4
- VOI's cranial area (tissue just ahead where the cylinder ends) n = 1

A gray intensity value (gray scale) was obtained from each VOI from the data obtained by the software, corresponding to the radiological density detected by the microCT device. Before each analysis, a correction of the gray intensity was performed with respect to the soft tissue of the sample where it has not been operated. All samples are normalized to the soft tissue of each micro CT uptake.

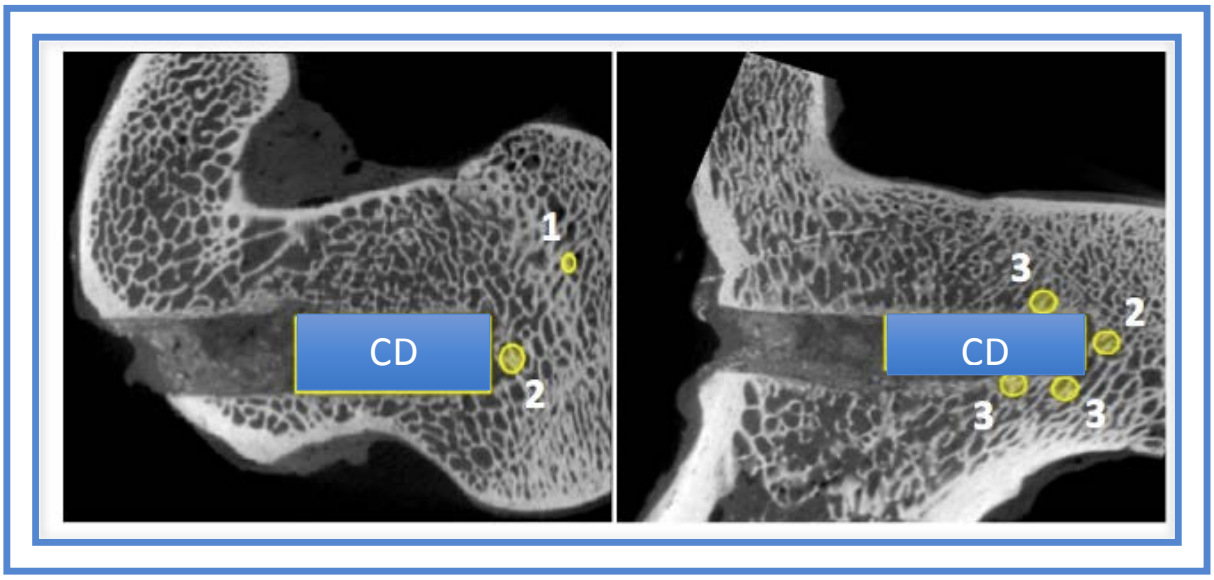


Figure 27. Axial and Antero-Posterior Micro CT-Scan views of the proximal femur showing the VOI distributions: 1. VOI Control zone (con), 2. VOI Cranial zone (cran), 3. VOIs Adjacent zone (are). CD: Core Decompression

12.7 Biomechanical evaluations

The biomechanical analysis consists in determining the compression modulus of the samples to be analyzed by means of mechanical tests of fresh compression. For this reason, the biomechanical tests were performed the same day of sheep euthanasia in order to avoid the immersion of the tissues in formalin and its consequent stiffening.

After the euthanasia of each sheep, the treated femoral head was removed for analysis and cut into two halves using an Exakt 310 diamond band (Exakt, Germany). One of the cut halves was used for the biomechanical test and the other half was immersed immediately in formalin for subsequent histological analysis.

All biomechanical tests were performed using a universal Bionix 358 servo-hydraulic mechanical test machine (MTS, USA) at a constant speed of 2mm / min (Figure 28). The indentations were performed using a cylindrical indenter of 4 mm diameter that was inserted inside the sample until a minimum distance of 1.5 mm of distance from the surface of the sample.

The biomechanical analyzes that were carried out consist of the realization of 4 indentations per sample through the application of a mechanical compression test (Figure 29). The 4 indentations were performed in 4 different zones previously established for all samples:

- HT zone (trabecular bone): healthy trabecular tissue of the femoral head that has not been exposed to any treatment or surgical procedure.

- Zone 1: final area of the canal made by the trocar, where the necrotic process of the tissue has occurred and the subsequent application of the repair treatment.

- Zone 2: inside the channel made by the trocar.

- Zone 3: start of the channel made by the trocar.

Data were acquired using the TestWorks software (MTS, USA) and were treated with Excel spreadsheets (Microsoft, USA).

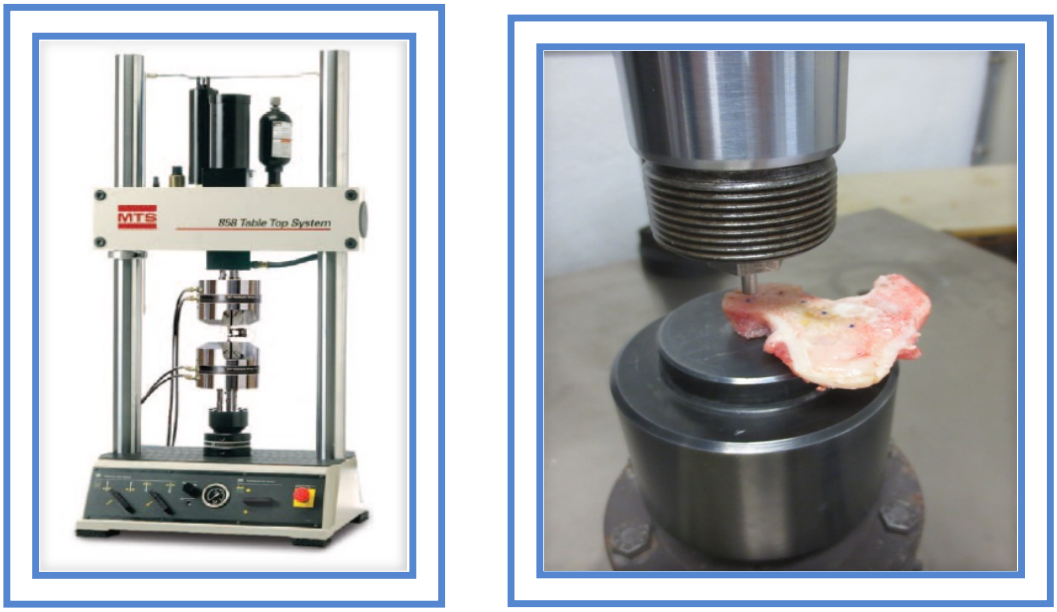


Figure 28. Bionix 358 servo-hydraulic mechanical test machine (MTS, USA)

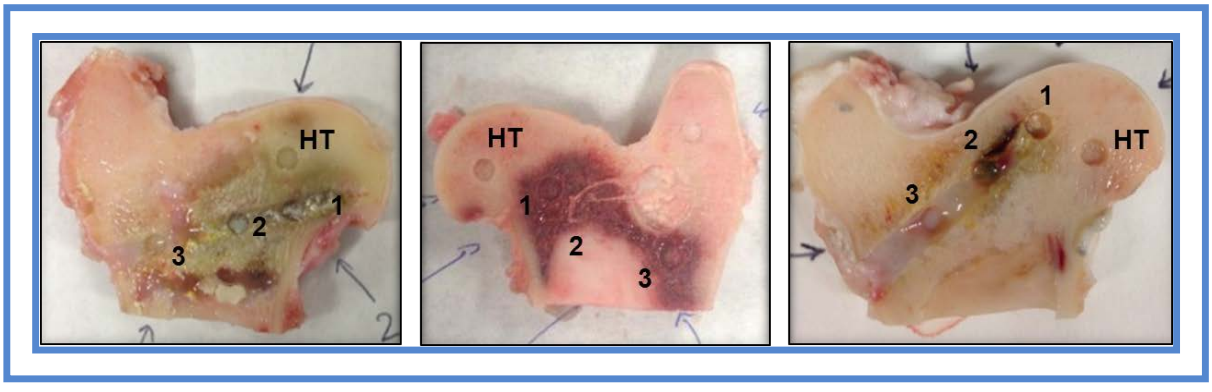


Figure 29. Distribution of the location of the indentation at the level of the femoral head

12.8 Histological technique:

The histological samples were processed by performing several cuts of the femoral head parallel to the longitudinal axis of the canal made for the introduction of the treatment in the previously osteonecrosis zone. The cuts were performed in order to obtain different thin sheets of approximately 2-3 mm thick from each femoral head (Figure 30). The objective of the realization of these cuts consisted in obtaining some sample in which the channel was observed along its longitudinal axis.

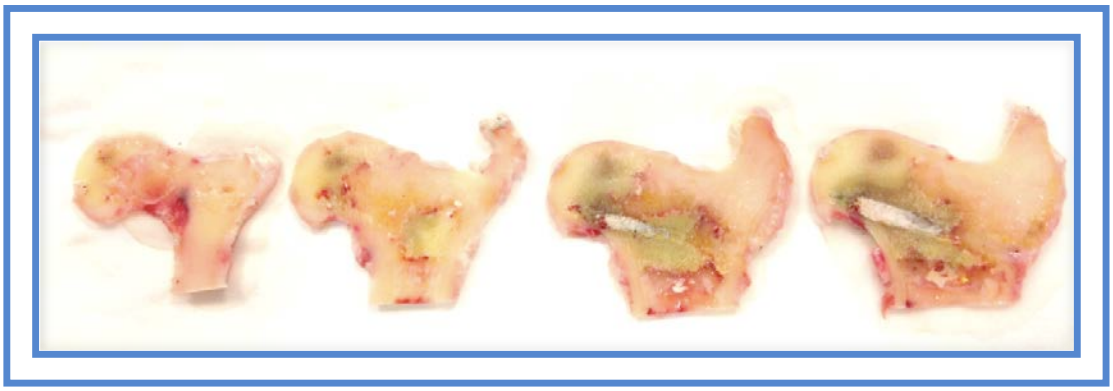


Figure 30. Longitudinal cuts of the femoral head.

The cuts were performed using an Exakt 310 diamond band saw (Exakt, Germany). The obtained cuts were immersed in a 10% solution of buffered formaldehyde for at least 1 week to ensure their fixation. Subsequently, the samples were subjected to a dehydration process, which was carried out by immersing the samples in a series of successive baths of ethanol in aqueous solution at different concentrations (30%, 50%, 75%, 96% and 100 %) with constant stirring (50 rpm).

After the complete dehydration, the process of inclusion in PMMA Technovit 7200 VLC resin (Kulzer-Heraus, Germany) was carried out by immersing the samples

in ethanol solutions with increasing concentrations of MMA resin (30%, 50%, 75%, 100%) under constant stirring (50 rpm). Samples submerged in 100% resin were solidified by the use of EXAKT photopolymerization equipment (Exakt, Germany) with white and UV light control unit (Figure 31).

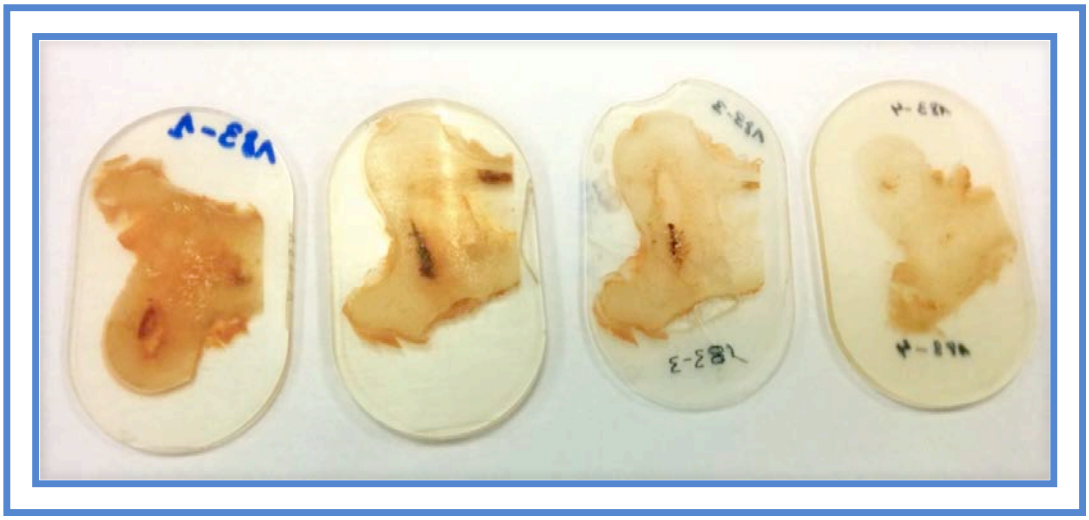


Figure 31: Images of the solid blocks of PMMA resin with the bone samples inside.

Samples already embedded in PMMA resin were polished until the surface was completely smooth and the tissue exposed. Polishing was performed with silicon carbide (SiC) abrasive papers using an Exakt 400 CS polisher with parallelism control. The polished surface was then adhered to an EXAKT PMMA histological plate by Technovit 7210 VLC (Kulzer-Heraus) monomer photo polymerization with an EXAKT ultraviolet light lamp.

Once fixed to this plate, another cut was made in order to remove the maximum amount of surplus tissue and resin to reduce the thickness of the sample. After the cut, the sample was again polished with abrasive (SiC) papers using an Exakt model 400 CS

polishers with parallelism control until a final sample thickness between 50 and 100 micrometers (counting sample and monomer). Taking successive measurements with the micrometer after each polishing period to know at all times the height of the sample monitored the thickness of the sample (Figure 32).

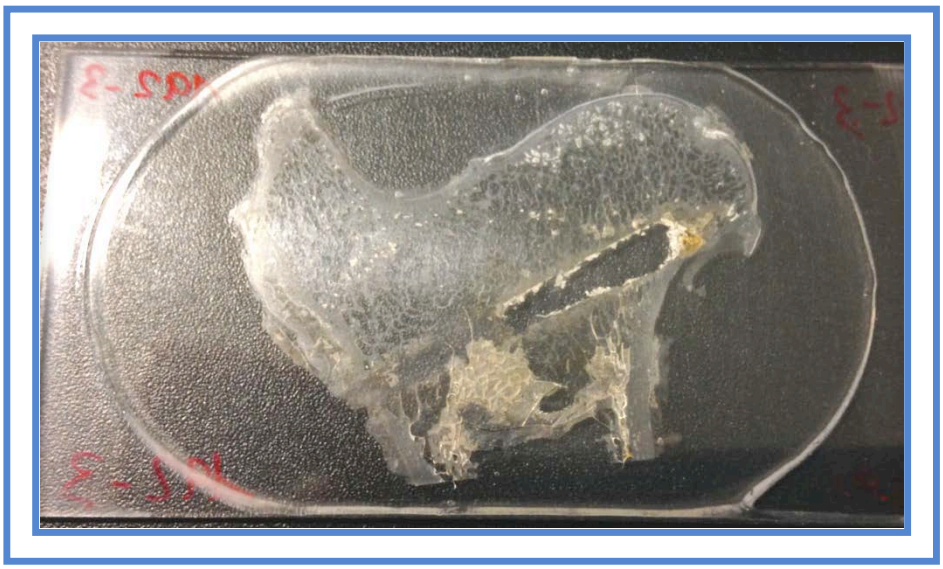


Figure 32: Final aspect of the sheet once polished up to 50-100 micrometers of diameter.

All samples were stained with Toluidine Blue staining, as it allows to observe the lines of cementation of the bone tissue as well as to differentiate the level of mineralization of the same.

Histological scoring evaluation:

We selected 12 non-decalcified histological preparations stained with Toluidine Blue in sagittal section (four femoral heads per experimental group). From each femoral head, four images of conventional optical microscopy were obtained at a 4x magnification of each previously established zone (Figure 33):

Zone 1: trabecular tissue of the femoral head remote from the core decompression channel and the experimental treatments implanted (n = 4).

Zone 3: tissue of the femoral head corresponding to the most proximal zone of the core decompression channel and to the implantation area of the experimental treatments (n = 4).

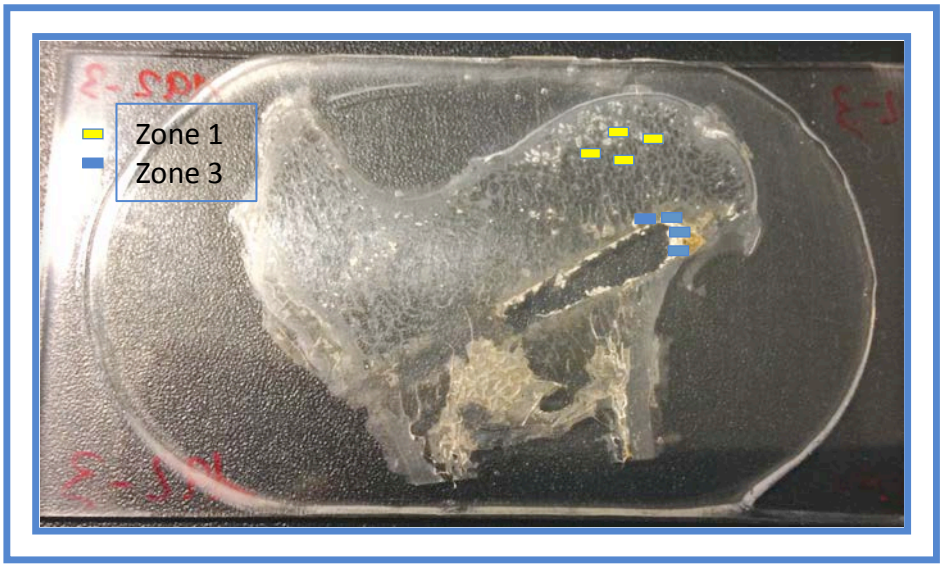


Figure 33: Image of the zones of the femoral head evaluated in the semiquantitative histological analysis.

All the images were examined by two independent observers and evaluated by a semiquantitative strategy adapted from the histopathological scoring parameters described by Udehiya *et al*. and by Simank *et al.* (Table 6). The averages of the defined parameters were calculated and studied in an independent way. It was considered a greater stimulation and bone regeneration in that or those groups with the highest score obtained.

Table 6: Histological Scoring System

Parameters of histological scoring		
Parameter		**Score**
1	***Osteogenesis***	
	No osteogenesis	0
	Weak Osteogenesis	1
	Medium Osteogenesis	2
	Good osteogenesis	3
	>75% of bone tissue is newly formed bone	4
2	***Trabecular Bone***	
	Without cellular bone activity	0
	Initial position of newly formed bone	1
	Active apposition of newly formed bone	2
	Reorganization of the trabecular bone	3
	Complete reorganization of the trabecular bone	4
3	***Trabecular bone resorption***	
	Intense resorption	0
	Moderate resorption	1
	Absence of resorption	2
4	***Bone Marrow***	
	Absent	0
	Start appearance	1
	Present in the middle of the trabecular space	2
	Complete colonization of the trabecular space	3
	Mature Bone Marrow	4

RESULTS

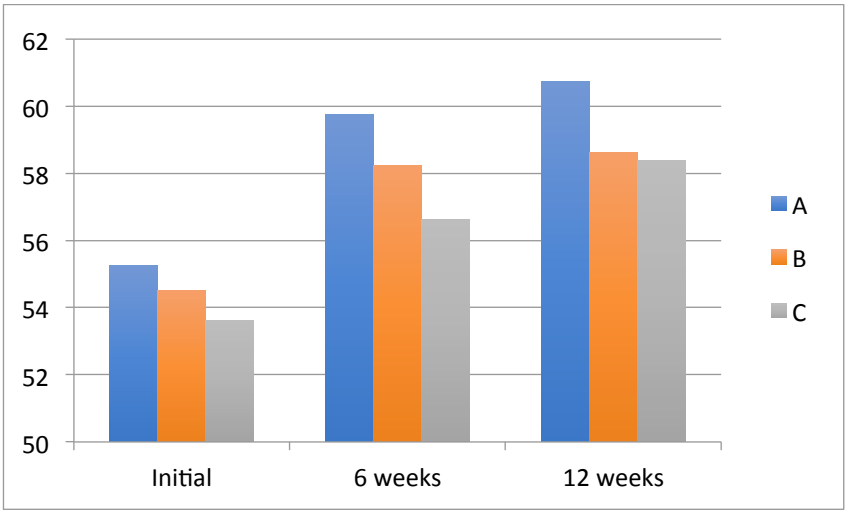
13. - Clinical Results

13.1 Weight

The percentage of weight variation during the treatment period (from week 6 to week 12) and throughout the experimental period (from week 0 to week 12) was studied. No statistically significant differences were observed between the groups during the treatment period (from week 6 to week 12). The overall weight difference (baseline weight at the end of the study at week 12) also did not change between groups.

Table 7: Distribution of weight by groups and temporality. The results are given in average weight (kg).

Group	Initial	6 Weeks	12 weeks
A	55,25 (48,50-60) Kg	59,75 (52,5-63) Kg	60,75 (56,50-66,50) Kg
B	54,5 (51-61,50) Kg	58,25 (55-61,50) Kg	58,625 (50-62,50) Kg
C	53,62 (48,50-59,50) Kg	56,625 (54-62,50) Kg	58,375 (53-63) Kg



13. -2 Complications:

We used in our experimental study 20 sheep in total. They were monitored daily throughout the study period, caregivers, technicians and research personnel involved, animal welfare advisers and veterinarians who assisted the animals carried out the observation of the animals. During the 24 hours a remote surveillance system was also available through a webcam that allowed the sheep to be recorded and evaluated. A 40% of major complications were found during the course of the study.

Two sheep from group A (β-TCP granules loaded with bone marrow concentrate) were found dead in postoperative period. First Sheep was found dead in the shed one day after the femoral head osteonecrosis induction surgery, no intraoperative incidence was reported. The second one was also found dead in the shed in the 14th day after treatment surgery. No intraoperative incidence was reported. In group B (β-TCP granules loaded with BMP-2) there were 4 major complications. Two sheep presented pulmonary embolism and were found dead in the second day of the treatment surgery. We confirm the diagnosis together with Veterinarians performing a necropsy study. The third sheep was found 10 days after treatment surgery lying unable to walk. An urgent X ray was done and a Subtrochanteric fracture was noticed (Figure 34). The sheep was euthanized and the proximal femur was resected, we noticed a fracture through the decompression canal (Figure 35). We considered that the decompression canal was to inferior related to the major trochanter and femoral neck. The other sheep presented a respiratory stress in the immediate postoperative period and 4 hours after the treatment surgery the animal died. In the Core Decompression group (Group C) one sheep suffered a Cardiorespiratory arrest during the anesthetic induction protocol before the treatment surgery. Resuscitation maneuvers were started including intracardiac

adrenaline and closed chest massage. Twenty minutes after the initiation of the maneuvers the sheep died. Another sheep was found dead in the shed 7 days after the femoral head osteonecrosis induction surgery. No intraoperative incidence was reported.

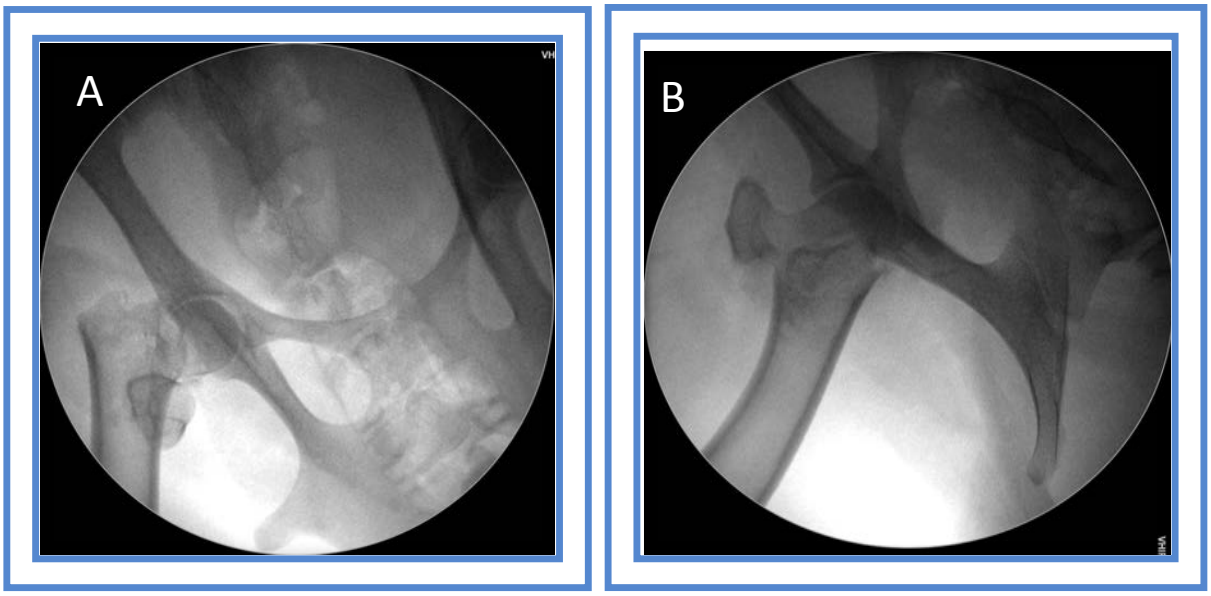


Figure 34. An AP and axial Xray view of the right hip showing a subtrochanteric fracture.

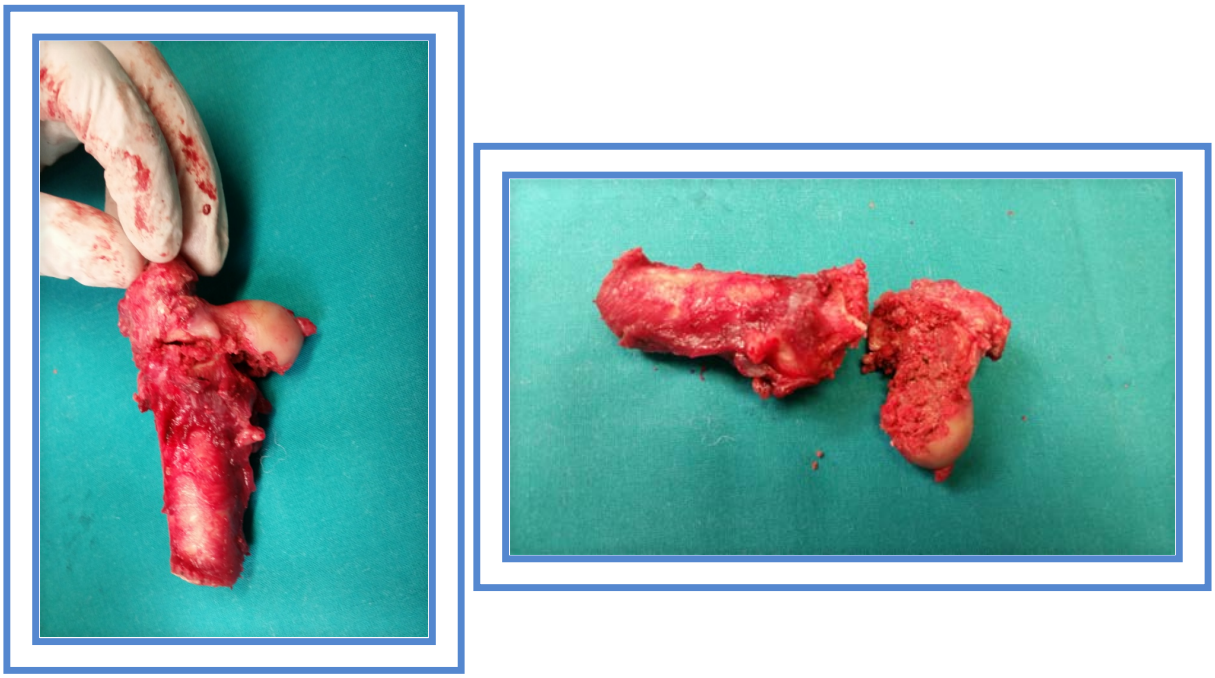


Figure 35: Anatomopathological piece of the right proximal femur with the subtrochanteric fracture.

14. - Animal model evaluation:

We used the cylindrical bone extracted with the trocar at the core decompression surgery in order to confirm the necrosis in the femoral head. Macroscopic evaluation of all the animals studied confirmed a necrotic bone tissue in the cylinder extracted (Figure 36). Furthermore a histological analysis was performed to all the cylindrical samples. It was confirmed the absence of osteocytes in the lacuna and fibrosis of the bone marrow (Figure 37,38). The middle longitudinal third of the cylinder presented progressively more viable bone with osteocytes in the lacuna, until reaching the more distal and lateral area where there was histology of normal bone. These areas of the cylinder corresponded with the division by areas of the femoral head between the ischemic, transitional and normal.

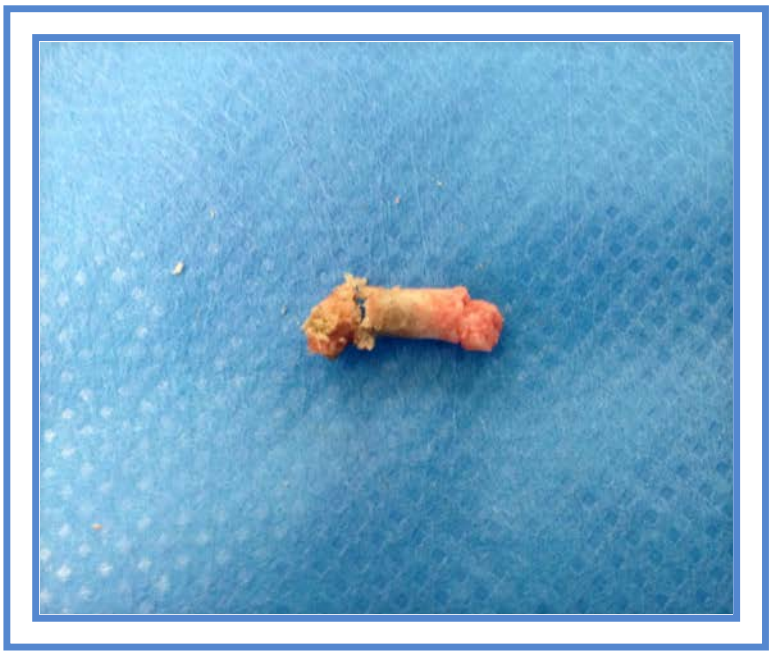


Figure 36: The bone cylinder extracted from the decompression surgery with a proximal necrotic zone.

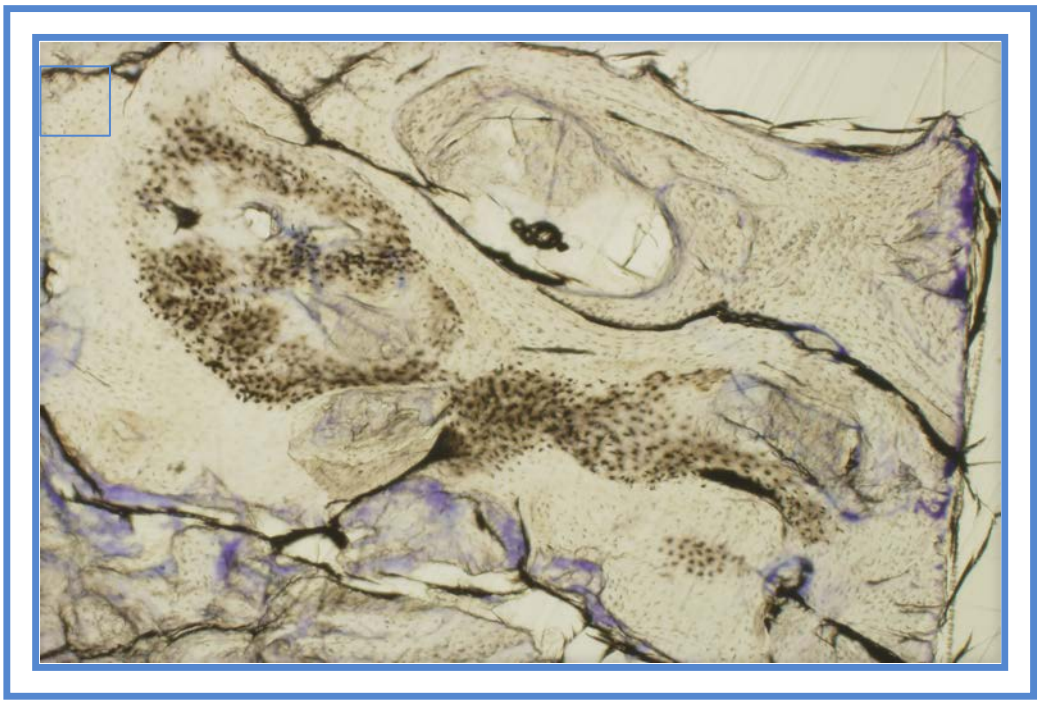


Figure 37: a 4x Histologic image of the bone cylinder showing necrotic changes with bone marrow fibrosis and empty lacunas.

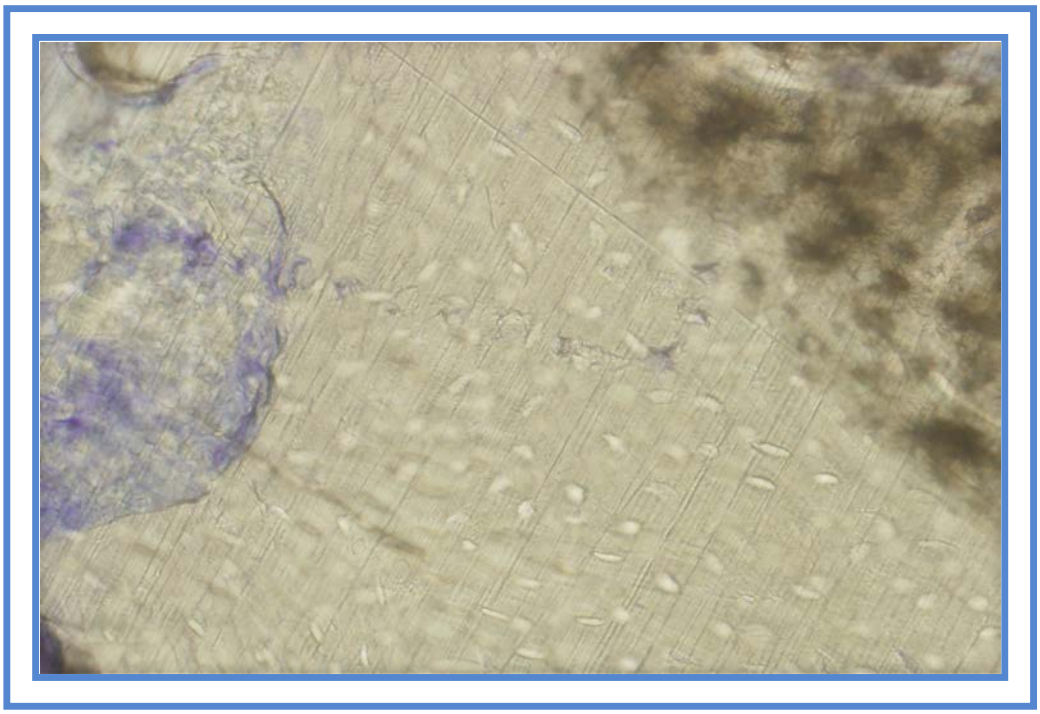


Figure 38: A 20x histologic image showing empty lacunas absence of osteocytes.

15. - Micro CT Scan

The gray scale values of the trabecular tissue in zone 1 (control) were very similar in the different groups without observing statistically significant differences between them (p> 0.05). The results are shown on table 8

Table 9 shows that both groups with β-TCP granules (Group A and B) had higher VOI`s values compared to Core Decompression (Group C) but no statistically significant differences were observed between the groups regarding bone density in the cranial zone of the femoral canal (p> 0.05).

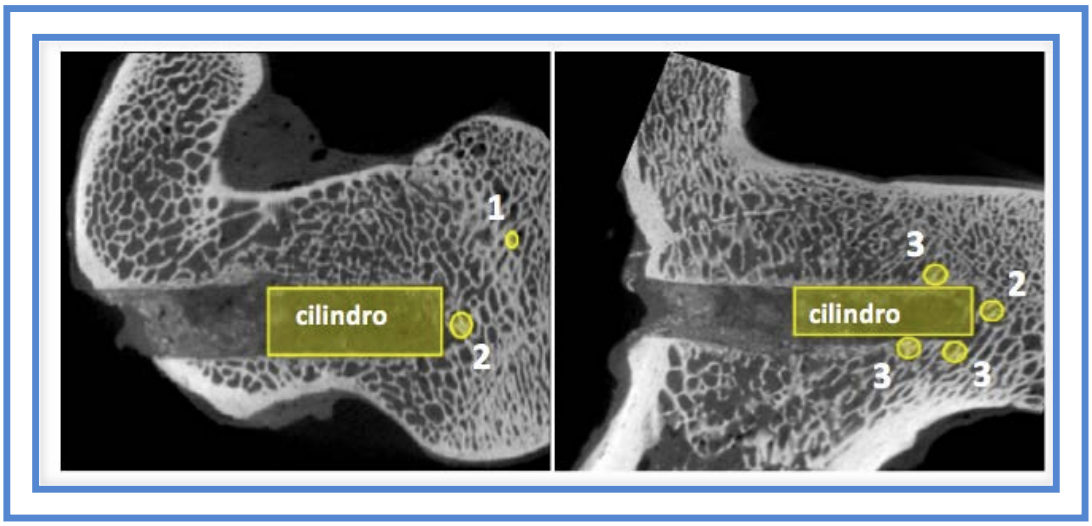


Figure 39: 1-VOI Control zone (con), 2-VOI cranial zone (cran), 3-VOIs Adjacent zone (are)

Table 8: Gray scale values in the control zone (zone 1) of the different treatment groups.

Groups (control zone 1)	Gray scale mean	Gray scale range
A	1293,826	(1087,236 - 1552,745)
B	1236,601	(970,486 - 1401,796)
C	1206,382	(938,0801- 1394,209)

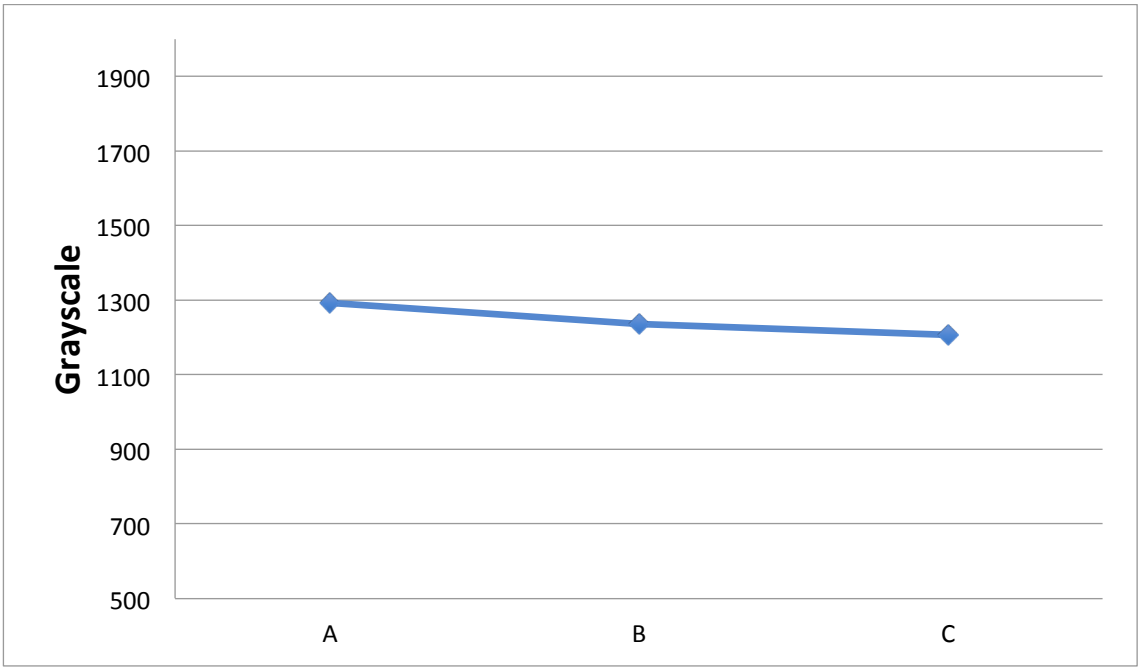
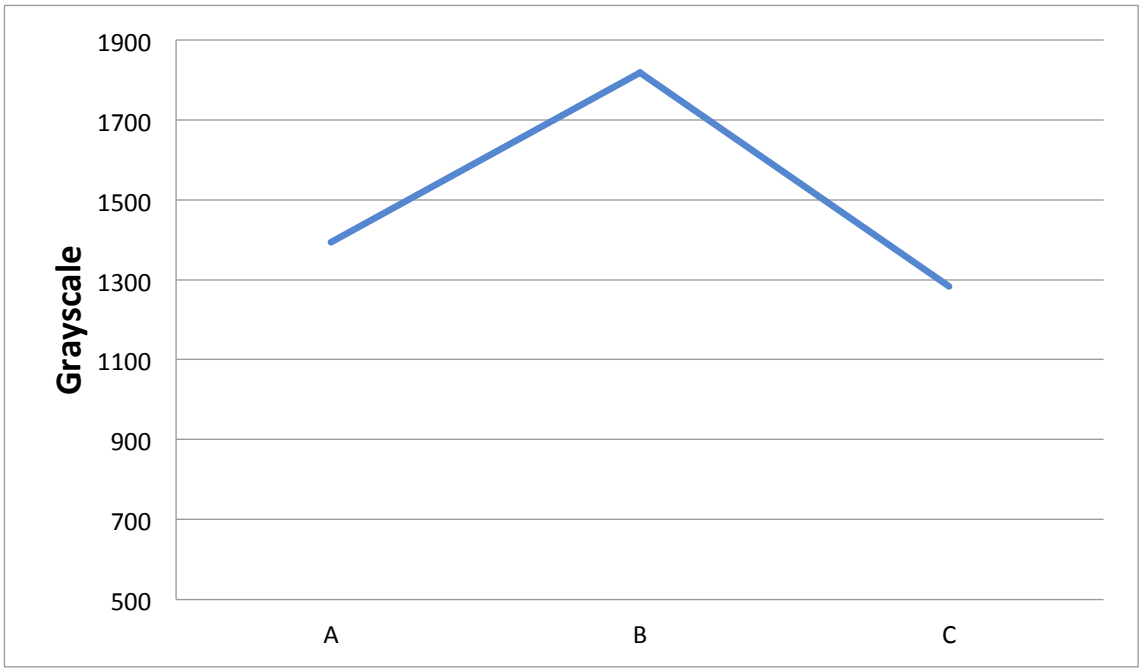


Table 9: Gray scale values in the cranial zone (zone 2) of the different treatment groups.

Groups (cranial zone 2)	Gray scale mean	Gray scale range
A	1393,660	(121,6744-2026,386)
B	1819,332	(1673,306- 1965,359)
C	1283,216	(834,2331- 1732,199)



16. - Biomechanics

The results obtained from the biomechanical analysis performed on the samples of groups A, B and C of the four zones are shown as a mean of the compression module obtained from the indented area in GPa (Table 10). The values obtained show that in zone 1, corresponding to the zone immediately adjacent to the implantation of the treatments they are minors with respect to the zone control of trabecular weave remote of the channel of central decompression. Zone 2 presents very low values due to the main presence of fibrous tissue by filling the central decompression channel. Zone 3 presents variability in registered values, probably due to the oblique orientation of the central decompression channel during the moment of the femoral head cut.

The results obtained in the biomechanical analysis do not present statistically significant differences between the groups ($p > 0.05$)

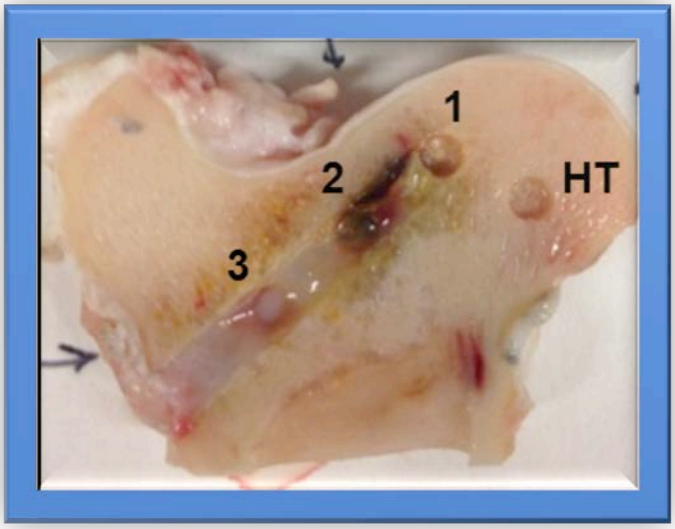
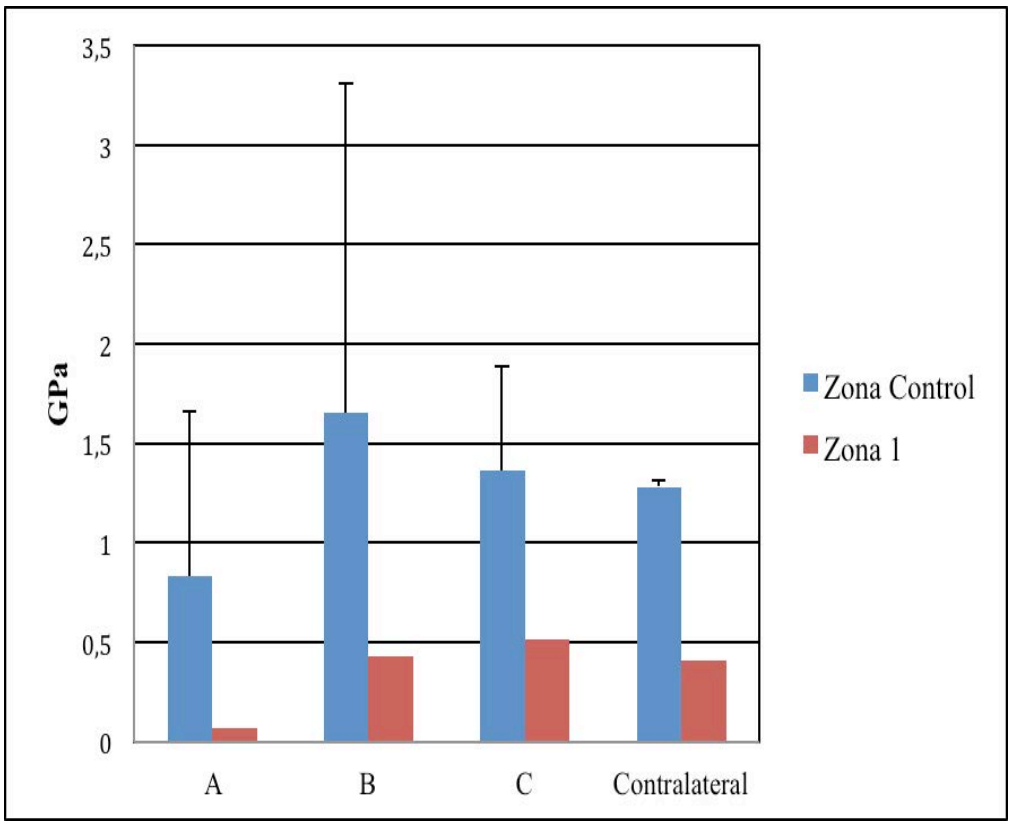


Figure 40: Distribution of the location of the indentation at the level of the femoral head

Table 10: Average of the compression module obtained in the control zone and zone 1 (adjacent to the implantation of the treatment) for groups A, B, C and contralateral femoral head.

Group	N	Control Zone	Zone 1	Zone 2	Zone 3
A	4	0.83 ± 0.83	0.07 ± 0.10	0.07 ± 0.05	0.29 ± 0.25
B	4	1.65 ± 1.66	0.43 ± 0.34	0.16 ± 0.16	0.58 ± 0.87
C	2	1.36 ± 0.53	0.51 ± 0.35	0.04 ± 0.06	0.02 ± 0.02
Contralateral	2	1.28 ± 0.03	0.41 ± 0.23	0.10 ± 0.15	0.22 ± 0.06



17. - Histology

17.1. -Toluidine Blue.

The proximal femur of the contralateral hip of the sheep included in the study was used to analyze the normal anatomy of the femur head. The normal structure formed by bone marrow without necrosis and bone lagoons filled with osteocytes (Figure 41). In all the analyzed samples the presence of bone remodeling was observed in the preexisting trabeculae. In group A and B β-TCP granules were observed in the process of resorption surrounded by osteoclastic material and limited formation of new trabeculae (Figure 42). In the control group (group C), greater bone regeneration was observed with the presence of neoformation of trabeculae with areas of apposition signs zones of a new layer of lamellar bone (Figure 43).

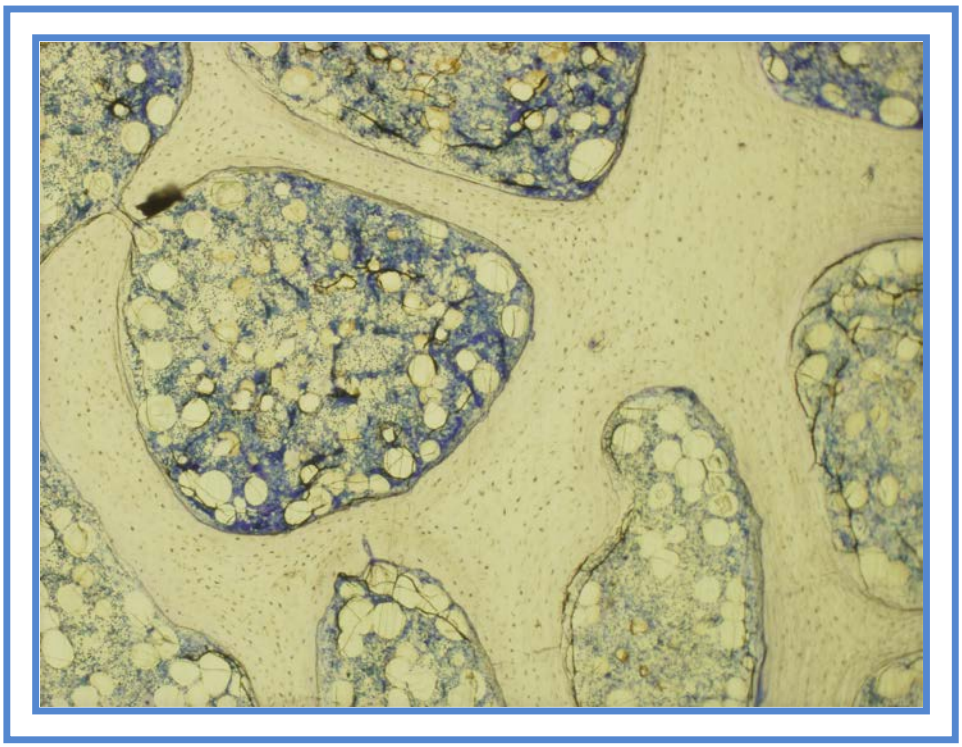


Figure 41: 4x magnification histology of the contralateral femoral head showing normal histological findings.

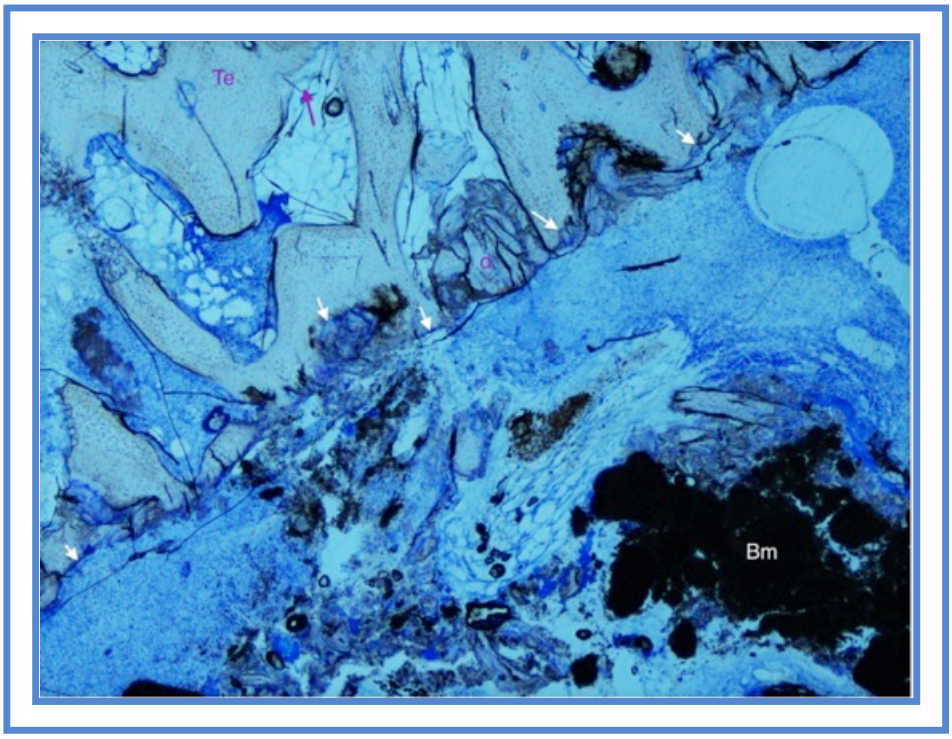


Figure 42: A 4x histologic image of a β-TCP group sheep showing intact granules (Bm), osteoclastic apposition (white arrows) and preexistent trabeculae (purple arrows).

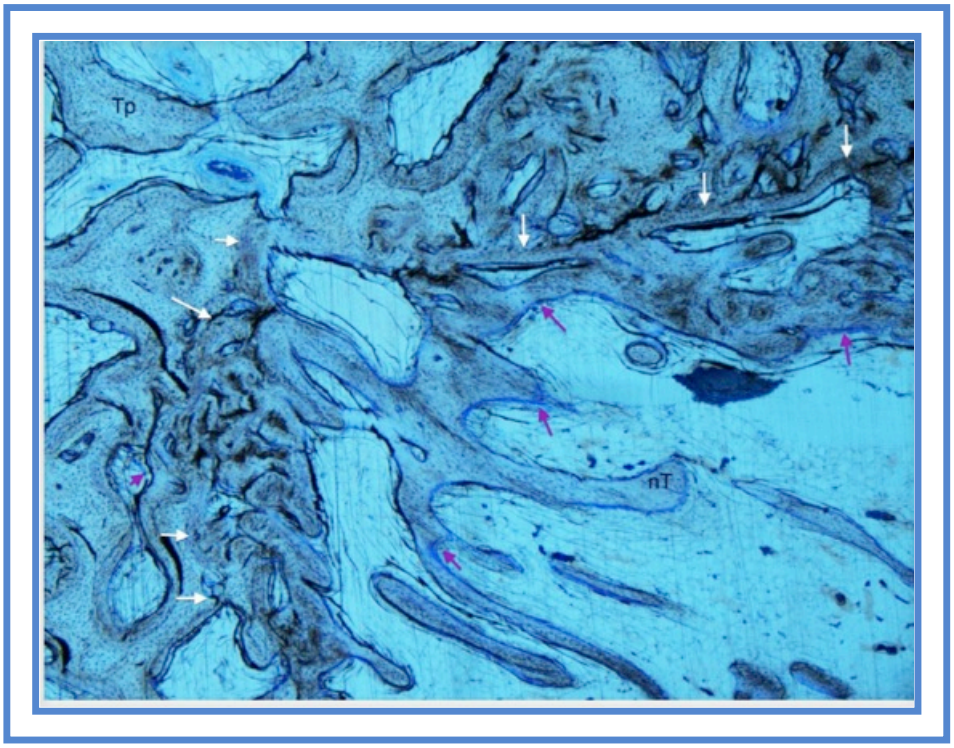


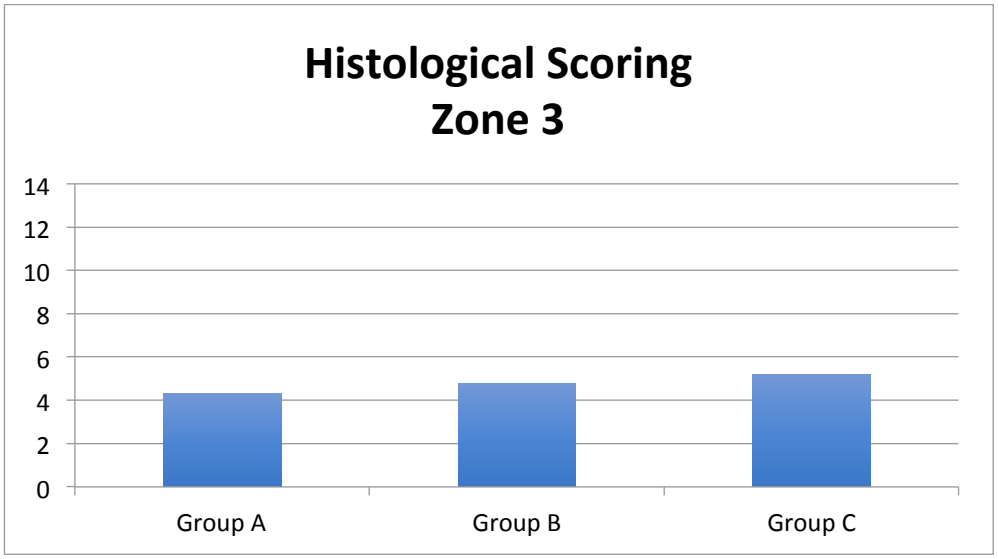
Figure 43: A 4x histologic image of a control group sheep showing mature neotrabeculae formation (purple arrows) and osteoclastic apposition (white arrows).

17.2. - Histological scoring evaluation:

In the semiquantitative analysis of histology using the scoring system established in our study, low scores were observed in all treatment groups, a result that corresponds to the subjective analysis of histology where, although signs of bone regeneration were observed, these were still precarious or in early stages. A higher score tendency was observed in the control group (group C) without statistically significant differences ($p > 0.05$). The results are shown in the table 11.

Table 11: Histological scoring values.

Group	Histological Score	
	Mean	Range
A (32)	4,3	(3,8-4,7)
B (32)	4,8	(4,0-5,2)
C (32)	5,2	(4,5-5,8)



DISCUSSION

18. - Preclinical Femoral Head Osteonecrosis Animal Model

In our study, we were able to establish a novel sheep model of femoral head osteonecrosis (ONFH) with the use of a minimally invasive and percutaneous helium-argon based cryotherapy system.

The development of experimental animal models of ONFH is indispensable in order to understand the etiology and pathophysiology of the disease, as well as to test new treatment modalities. Clinical trials of novel treatments for osteonecrosis (ON) have been impeded by the lack of an appropriate experimental animal model of the human disease. Experimental animal models of ONFH have been the subject of a number of studies, with various animals being used, including small and large biped and quadrupeds (55). When designing an experimental animal study it is essential to correctly establish the objective of the research and then choose the most appropriate animal (174).

18.1. - Animal Selection

In our study, we decided to use sheep as an animal model. As sheep are large animals, their hip anatomy resembles that of human beings. This means that the results can be transferred to our clinical practice (175). Another advantage is that they are docile and easy to house and manage. Our research team also has extensive experience in the management of the sheep, established over time and over various experimental studies (176–178).

Several animals—including rats, dogs, rabbits, sheep, goats, and emus—have been used as models, both to study the pathogenesis ONFH and to test different treatments for the disease (137,139–141,179,180). Rats, being the first animals domesticated for purely scientific reasons, are the animals of choice in most studies that analyze the pathogenesis and risk factors of ONFH (57,145). However, we considered that since they are small animals and have very different bone composition and regeneration capacity to humans (181), they are not ideal models for analyzing different types of treatment. Some authors have also used dogs as animal models of ONFH (139,182,183). Theoretically, dogs provide ideal models for the study of bone regeneration, and out of the animals most commonly used in experimentation; dogs are those that have the most similar bone composition to that of humans (181). For this reason, our research group discussed the possibility of using dogs in our study, but, given the sociocultural implications arising from the fact that dogs are commonly kept as pets, we opted for the use of sheep instead.

Some quadruped animal models of ON (184–186) have failed to progress to end stage mechanical collapse and it has been thought that this failure may be related to the influence of limb weight-bearing on the development of ONFH. One of the studies with the greatest impact on the development of animal models of ONFH was published by Conzemius *et al.* (137). This study used emus as experimental animals because they are birds with a bipedal gait, and it concluded that this could contribute to the establishment of a model that more accurately simulates all phases of pathology in humans. A study using an animal model developed from African ostriches has also been recently published (187). The authors established an animal model of ONFH and observed in all the animals the collapse of the femoral head; they concluded that, as ostriches have a

bipedal gait like humans, they were able to produce all the phases of the entity. Nevertheless, in our study, the uses of emus or ostriches was excluded because both experimental animals have little history in preclinical research and are not available in Europe for animal studies. Furthermore, Conzemius (137) warned that emus can be aggressive and more difficult to house and manage. Questioning this weight-bearing theory, Okazaki *et al.* (188) established an animal model based on rats and compared the effect of weight-bearing on the development of ONFH. They did not find any differences between the weight- and non-weight-bearing groups and concluded that weight bearing is not related to the development of non-traumatic ONFH. We consider that, in spite of the above-mentioned studies, the relationship between weight bearing and the development of ONFH in animal models is not well established and the most important factor is probably the mechanism of necrosis induction. Using quadrupeds sheep in our study, we were able to establish a well-defined and irreversible necrotic lesion in the femoral head, regardless of weight bearing and without achieving the collapse of the femoral head. This is the phase of the disease where there is less evidence for the ideal treatment.

18.2. - Selection of the femoral head osteonecrosis induction mechanism

Experimental studies related to the ONFH induction mechanism in animal models are, in general terms, divided into traumatic and non-traumatic models (137,189). Although non-traumatic models have been used in different studies to evaluate possible etiologies related to ONFH and to try to understand the pathophysiology of the disease, traumatic models are more useful when comparing

different treatment options because the necrotic lesions are concentrated in the femoral head and a complete range of ON stages can be induced (149).

Based on the previous experience of other authors (137,141,190,191) in our study we decided to use a traumatic mechanism known as thermal insult. With the objective of improving, optimizing, and refining the techniques previously described by other authors, we also decided to use a minimally invasive percutaneous helium-argon based cryotherapy system to induce a pre-collapse stage of ONFH. At six weeks, we were able to induce pre-collapse ONFH in all animals included in the study. The cryoprobe was fundamental in various aspects of technique refinement and objectivity, and it permitted correct placement and verification with intraoperative image intensification. The cryoprobe (Cryo-44 Crioprobe, Endocare Cryocare, HealthTronics) used in our study is a commercial product used in many medical areas, including general surgery, urology, gynecology, oncology, neurology, dermatology, proctology, pulmonary surgery, and thoracic surgery. It delivers cold temperatures to tissue intended for therapy and warm temperatures to tissue that needs to be thawed. The cooling and warming capacity is limited only to the distal end of the cryoprobe. The system also has a display where one can set the temperature and time protocol. These characteristics make our ON model objective, constant, and reproducible.

Conezemius *et al.* (137) were pioneers in using thermal insult as an ONFH induction model. In their study, an ONFH model was established by cryogenic insult with a direct stream of liquid nitrogen delivered into the cancellous bone of the femoral head through a foramen located near the head-neck junction, combined with vascular ligation. Compared to our method, it has many disadvantages. Making a perforation in

the femoral neck to introduce liquid nitrogen increases the risk of subcapital fracture of the femur. Furthermore, neither the dosage of the liquid nitrogen nor the temperature reached in the administration zone can be accurately measured. This can cause an injury to the wider femoral head and may have implications when comparing the different therapeutic modalities.

The Conzemius technique, described with some modifications, is the ONFH induction mechanism most widely used by other authors for the traumatic induction of ONFH (141,190,191). Reed *et al.* (192) designed a cryoprobe that was placed under radiographic guidance into the subchondral bones to create necrosis of the target area. Goetz performed a finite element analysis on the necrotic area induced by this device and confirmed the reliability of this technique. This method, compared to the one used in our study, has the disadvantage of not being reproducible because the cryoprobe used was their own design and is not commercially available. Velez (141) employed a commercial probe—Brymill Cry-AC—that was based on the Conzemius method and used the Reed modifications. It was a 2 mm cryoprobe that reached a temperature of -196 °C at its tip, but it did not offer data on the temperatures reached after intraosseous application. In our proposed animal model, a 2.4 mm cryoprobe was used. It creates a circular necrotic lesion with a radius of 2 cm and also allows accurate monitoring of the temperature reached by the tip of the probe. The use of this cryotherapy probe represents an advance in ONFH induction mechanisms and enables the creation of a homogeneous, reproducible, and less invasive model.

The idea of combining freezing and heating with the purpose of necrotizing malignant tumor tissues was first proposed in 1982 (193). The freezing process causes

direct cellular and vascular injury. Meanwhile, in hyperthermia treatment, the moderate increase of tissue temperature for a certain time period is expected to induce cell death by affecting membrane fluidity and cytoskeletal, protein, and nuclear structures, while also disrupting DNA replication (138). Liu T *et al.* (194) designed a cryoprobe system with vapor heating and found that freezing immediately followed by rapid heating of the target tissue could improve the treatment effect due to thermal stress.

Basing ourselves on the theory described above, we decided to use a system that would allow us to induce a thermal necrotic lesion by alternating cycles of cold and heat. After our study had begun, Wang *et al.* (142) published a study first on rabbits and then on dogs (182), using a helium and argon system for the induction of ONFH. Unlike in our study, they used nuclear magnetic resonance as a guide for the intracephalic placement of the probe. They compared the need to perform one or two cycles of cold and heat for the inducement of ONFH and observed that the percentage of empty lacunas in the histology was higher in the group of two cycles at both four and eight weeks. These findings coincide with the results of our animal model, where we observed an irreversible necrotic lesion at six weeks after the induction of necrosis using two repeated cycles of cold and heat. On the other hand, Poignard *et al.* (195) have carried out an animal model study with pigs, comparing a cryogenic insult with repeated freeze-thaw cycle associated or not with a ligature of the posterior circumflex. They observed that at eight weeks there was complete bone regeneration in the cryogenic insult group without vascular ligature. Furthermore, they concluded that cryoinjury associated with partial vascular coagulation is sufficient for obtaining localized and sustainable necrosis in the subchondral area of the femoral head, reproducing all stages of the human disorder. The results from Wang *et al.* (142) and

Poignard *et al.* (195) confirm our proposal of a new pre-collapse ON animal model with sheep and also call into question the routine use of vascular ligation in the induction of femoral head necrosis, which is generally associated with morbidity.

18.3. - Animal model evaluation and complications

Although in clinical practice MRI is the gold standard test for the diagnosis of ONFH (196–198) especially in pre-collapse stages, most of the studies related to experimental animal models have used histological analysis to confirm the diagnosis of ONFH (137,139,140,199).

In our study, we decided to use the bone cylinder obtained from core decompression surgery for histological analysis to confirm the necrotic lesion. Our histological findings at six weeks of the evolution of ON were consistent in all the samples: fibrotic bone marrow, empty lacunas, and unstructured trabecular architecture. It is the first study to use the cylinders obtained in surgery to confirm the diagnosis of ONFH. We decided to use the cylinders because we consider that the definitive diagnosis of the entity is made by histology, which in turn also allows us to reduce the number of animals needed for the study and comply with the principle of reduction of animal research (200). Our histology results agree with the results obtained by Manggold *et al.* (140) they observed that at six weeks more than 80% of the lacunas were empty and the majority of the bone marrow were replaced by fibrotic tissue. Conzemuis *et al.* (137) also found at six weeks that normal marrow was replaced by fibrotic marrow and osteocyte-filled lacunas were replaced by empty lacunas. In order to simulate all phases of ONFH up to joint collapse, several authors have prolonged the follow-up to 12 weeks (142,176), 16 weeks (191), and even 24 weeks (187). With the

use of MRI and microCT, all studies to a greater or lesser degree manage to diagnose ONFH in its last stages of fracture and subchondral collapse. Guided by the results of other authors, in our study we decided to follow the sheep for no more than six weeks. We also did so because our initial objective in the study was to be able to induce a necrotic lesion in the femoral head without generating joint collapse, and because we consider it to be the best way to compare different treatment options in the phase of the disease where there is more controversy about the ideal treatment.

A brief mention should be made of the complications related to the animal model. It is surprising that most published studies authors do not comment in their results on the complications presented during the development of the experiment. 40% of major complications were found during the course of our study. The vast majority of the complications were related to respiratory problems. We had only one surgery-related complication a subtrochanteric fracture that was diagnosed 10 days after decompression surgery. We considered this to be a neglected complication because the decompression canal was too inferior in relation to the major trochanter and femoral neck. The fracture pattern observed in our study differs from the fractures observed in the initial Conzemius study, where the emus presented a femoral neck fracture rate of 22% (137). In their technique, they describe making a bone perforation at the level of the femoral neck, which enabled them to insert the liquid nitrogen that consequently weakened the area and contributed to the observed fractures. J. Manggold *et al.* (140) reported 20% of complications in their study with an ON model using sheep; they had one pulmonary embolism and one femoral neck fracture.

19. - Bone regeneration in Femoral Head Osteonecrosis

The post-collapse stages of ONFH have an effective treatment with good long-term results hip arthroplasty (201). However, there is insufficient scientific evidence to support routine use of a particular treatment for the pre-collapse stages, although many surgical treatments have been attempted.

There is a current trend of using biological treatment and bone tissue engineering for the pre-collapse stage of ONFH (3,155,160,161). Due to the previous experience of our research group in treating ONFH with biological therapy and the current trend of treatment with biomaterials, we thought that the core decompression associated with modern techniques of tissue engineering contribution in early stages of ONFH lesions was most appropriate for the design of new bone regeneration therapies.

19.1. - Treatment modalities selection

Although the pathogenesis of ONFH is uncertain, it is known that in the final phases there is an interruption of the blood flow in a segment of the head of the femur that produces cell death and that in many cases the repair process fails (202). Based on the hypothesis that ONFH has a cellular origin, treatments incorporating cell-based therapy have great potential (203). Lindholm and Urist (204) first described the use of unprocessed bone marrow aspirate with allograft bone matrix to enhance bone healing. The injection of autologous bone marrow-derived cells into the femoral head during early-stage ONFH was proposed by Hernigou *et al.* (122). Adult mesenchymal stromal cells can be isolated from bone marrow (205). These cells have multipotential capacity for differentiation into osteoblasts (206). Bone-marrow-derived mononuclear cells also promote formation of new blood vessels due to the presence of endothelial cell

progenitors or hemangioblasts in the bone marrow concentrate (207). Angiogenesis may be promoted by both the increased supply of progenitor cells and the angiogenic cytokines produced by the bone marrow cells. MSCs can also release a variety of growth factors to facilitate tissue regeneration in the microenvironment. Due to the above-described characteristics of mesenchymal cells, the ease of obtaining and processing the bone marrow concentrate, and the growing popularity of bone marrow usage (208), we considered it a very attractive option for research and decided to associate it with core decompression in one of our treatment groups (Group A), which comprised of four sheep.

Based on the cellular theory in the pathogenesis of ONFH, growth factors such as bone morphogenetic proteins (BMPs) also represent an attractive option for the treatment of this entity because they have the ability to initiate new bone formation by recruiting mesenchymal stem cells and stimulating their differentiation into osteoprogenitor cells (209). The osteoinductive properties of endogenous BMPs derived from the bones of numerous mammalian species have been characterized (175). Use of endogenous human or bovine BMPs as adjuncts for the successful treatment of recalcitrant long bone nonunion and segmental defects in humans has also been reported (210,211). The use of endogenous BMPs has not become popular due to its prolonged purification process, low amount of active substance, and risk of disease transmission (212). In the mid-1980s, scientists developed the means of producing BMPs via recombinant DNA technology, whereby the proteins were synthesized by bacteria (for example, Escherichia coli) or other cell lines (for example, Chinese hamster ovary cells) that had been transfected with a growth factor gene (213). As many as 20 BMPs have been identified. In our study, we decided to use rhBMP2 as a treatment in Group B once

core decompression was performed because, together with rhBMP7, it is commercially available and approved for use in medical practice. It has been demonstrated that it is an osteoinductive growth factor (214) and previous research has also suggested that recombinant rhBMP-2 may induce bone formation and osteoblastic differentiation by regulating endochondral ossification (215,216). It has also been demonstrated recently that it is beneficial for the treatment of ONFH because it promotes the expression of proangiogenic factors in synovial cells (217).

We decided to perform core decompression for all study groups, since it is currently the surgical method of choice for the pre-collapse phases of the entity (218) and because it enables the comparison of the results of different treatments in a more homogeneous way. This mode of therapy aims to lower the elevated levels of intraosseous pressure and enhance repair capacities at the site of necrosis via stimulation of neovascularization (219). In addition, it serves as a procedure prior to the application of adjuvant treatments. There are two methods of performing central decompression that differ in the diameter of the channels used. The most commonly used is a single, large perforation that provides the advantage of being able to debride the necrotic area (119). The other described method consists of making multiple perforations of smaller diameter in order to reduce the physical aggression to the femoral head, which can favor its collapse (220). In our study, we decided to use the single perforation method with a trephine of 5 mm. This allowed us to debride the necrotic lesion and at the same time use the trephine once it had been inserted into the area of the lesion to apply the various biomaterial treatments. The size of the trephine (5 mm) used in our study is the same as in previous studies published on the sheep animal model of ONFH (141,221). We decided to perform the core decompression technique without additional treatment on

four sheep (Group C) and cataloged them as the control group, which served as a basis for comparing the results obtained in the two remaining groups.

19.2. - Bioengineered scaffold selection

A very important aspect when performing a biological treatment with cell-based therapy is the choice of a carrier that serves as structural support and osteoconduction. Clinically, the most common strategy is the use of bone autografts. However, while biologically ideal, they present significant drawbacks, such as the need for a second surgical intervention, a limited harvestable amount and, occasionally, residual pain over time at the harvesting site (222). Grafts from bone banks or other animal species are still subject to risks such as immunological reactions or disease transmission (223).

We decided to use a bioceramic as a scaffold, mixing it with both bone marrow concentrate and rhBMP2 in Groups A and B respectively. Bioceramics are the most used biomaterials in tissue engineering for bone applications, and out of them we opted for calcium orthophosphate (β-TCP) due to its similarity to the mineral phase of bone (224,225).

The β-TCP microspheres used in our study also meet the requirements of an ideal scaffold since they provide an environment suitable for tissue development. β-TCP has good biocompatibility, with no detrimental effects on the surrounding tissues, and allows cell attachment, growth, and subsequent differentiation (226). It is also preferred for vascular remodeling because it is both osteoinductive and osteoconductive, and possesses the ability to osteointegrate. The latter ability means that it is gradually replaced by newly formed bone and is able to adapt to irregularly shaped defects and support mechanical load. It is also be storable and sterilizable without loss of its

properties (227)(228).

Collagen as a carrier for cell-based therapy has attracted attention in recent years due to its excellent biocompatibility, degradation into physiological end products, and appropriate interactions with cells and other macromolecules (229). However, collagen's inability to maintain space, leading to resorption of newly formed bone, can be one of its major disadvantages (230). One study has reported that collagen is not an ideal carrier of rhBMP-2 because it can degrade within seven days in vivo, reducing the biological effects of rhBMP-2 (231).

The porous structure of the ceramic contributed to the increased surface area and improved BMP-2 release into the areas surrounding cells. Porosity also accelerated the growth of osteoblasts by providing space for new bone formation. β-TCP has demonstrated excellent biocompatibility and bone conduction and has typically been shown to support attachment, differentiation, and proliferation of related cells (osteoblasts and mesenchymal stem cells) (232–234). An additional advantage is that β-TCP exhibits a faster degradation rate than crystalline hydroxyl-apatite (235). For the present study, the Department of Biomaterials, Biomechanics and Tissue Engineering of the Technical University of Catalonia – Barcelona Tech (UPC), synthesized spherical granules of β-TCP.

19.3. - Femoral head osteonecrosis treatment evaluation

In our sheep animal model of ONFH, once the samples were analyzed using a biomechanical study, microCT, and histology we were able to affirm that stimulation of bone regeneration was observed in all groups, with no significant differences between the cell therapy groups and the core decompression group.

Despite the fact that hematoxylin-eosin staining is used in most studies to evaluate the effect of treatments on bone regeneration in ONFH (176,221,236,237) in our study, following the advice of our team of pathologists, we decided to use toluidine blue staining. This stain has the advantage that, unlike HE, it distinguishes between new and existing lamellar bone, as well as distinguishing between metachromasia and chondroid tissue, resulting in a better morphological definition of calcified tissues involved in remodeling / regeneration (238). Some studies evaluating different treatments for ONFH have performed a qualitative analysis of histology (176,239), while others have performed a quantitative analysis by using software that allows one to count the percentage of new bone formed (190). In our study, we decided to establish a scoring system for the histology analysis of different treatments. We also decided to use as a reference the scoring systems proposed by Simank *et al.* (221) and Udehiya *et al.* (240) and we established a system with four sections that we considered relevant in the assessment of bone regeneration: osteogenesis, trabecular bone, trabecular bone resorption, and bone marrow. This system has a maximum score of 14 points that would correspond to a normal bone (Figure). To increase the sample size, we decided to take different photos both in the control area and in the area adjacent to the decompression channel in each studied section. Despite this and the use of the scoring system, we could not observe any clinically statistical differences between the treatment groups with respect to the control (core decompression).

Surprisingly, in the analysis of the histology stains, it was observed that the sheep in the core decompression group (Group C) showed changes compatible with bone regeneration in more advanced phase than the groups treated with β-TCP (Group

A and B). We consider that these findings may be related to the degradation of β-TCP granules. In our study, it was observed that at six weeks the granules remained intact in the implantation area. The role played by degradation of calcium phosphates in osteoinduction is still unknown (241). Some authors claim that, while a certain degree of degradation is desirable because it provides the calcium and phosphate ions needed to stimulate MSC differentiation and foster mineralization, excessive degradation may impair osteoinduction due to the lack of a stable surface upon which new bone can be deposited (242). In a recent study that compared different scaffolds, it was demonstrated that the scaffold with the highest osteoinduction capacity was that which showed the highest degradation (241). The authors of the study also observed that the degradation of β-TCP granules occurred at 12 weeks. These findings may explain why we could not observe differences in bone regeneration between the treatment groups, since it was confirmed that the (β-TCP) granules were still in the absorption phase when histological analyses of the animals were performed six weeks after treatment.

The results of the biomechanical study agree with those obtained in the histological analysis. A trend of higher values was observed in the compression module in the sheep belonging to the core decompression group. This trend is believed to be due to the increased formation of new trabecular tissue in the most proximal zone of the decompression channel, resulting in a structure with greater resistance than the β-TCP granules that remained intact in the most proximal zone in the cell therapy groups.

Our study is one of the few experimental studies where microCT has been used to assess the outcome of treatments for ONFH. The use of microCT offers us information related to bone formation in a more quantitative way. In the analysis of the

microCT images, it was observed that, unlike the results of histological and biomechanical analyses, the β-TCP granule treatment groups (Groups A and B) had a gray scale volume higher than the core decompression group. We consider that this finding may also be related to the intact presence of the granules when the microCT study was carried out at six weeks after ONFH treatment.

Our findings differ from those obtained by Tang *et al.* (190) in an experimental ONFH study with goats. They divided the animals into three different groups. After core decompression, porous β-TCP loaded with the BMP-2 gene or β-galactosidase (gal)-gene-transduced BMSCs were implanted into the left and right femoral heads, respectively. At 16 weeks after implantation, there was collapse of the femoral head in the untreated group but not in the BMP-2 or β-gal groups. The femoral heads in the BMP-2 gene-modified bone marrow stem cells group had a normal density and surface, while those in the β-gal group presented with a low density and an irregular surface. Histologically, new bone and fibrous tissue was formed in the macropores of the β-TCP. These findings confirm the role played by cell therapy and biomaterials in the initial phases of ONFH and also support the importance of the degradation of β-TCP granules in bone regeneration. For us, this study has the limitation of not performing any type of treatment in the control group, which can lead to bias. We decided to perform a core decompression technique as a primary intervention for all animals to look for more homogeneity in the groups as well as to be able to make the comparison and observe the effect in the bone regeneration of the biological and tissue engineering treatments.

In another experimental study of ONFH in sheep Simank *et al.* (221) demonstrated that at 12 weeks after treatment with both BMP2 and GDF-5, the sheep

had higher bone regeneration compared with a control group that contained only a carrier. In their study, unlike ours, they decided to use polyglactin filaments as scaffolds for all the groups. Polyglactin has been demonstrated to have a rapid degradation capacity in vivo, and it can lead to local accumulation of lactic and glycolic acid, thus impairing cell growth and differentiation, as well as causing inflammatory reactions (243,244). For this reason, we consider that polyglactin is not an ideal scaffold for the treatment of ONFH with advanced cell therapy.

More recent studies have combined the use of mesenchymal cells of the bone marrow with a growth factor such as BMP2 associated with a synthetic bone scaffold. In a study with rabbits, Zeng *et al.* (237) divided the animals into three groups, one control group that did not undergo any treatment, another group with BMP2 and seeded bio-derived bone materials (BBM), and a third group with mesenchymal cells derived from bone marrow. The authors demonstrated that regeneration of new bone was observed in the histology at 12 weeks of follow-up only when the three elements (bone marrow, BMP2 and BBM) were combined. Subsequently, in the same research line, Zhang *et al.* (236) generated a novel calcium phosphate composite scaffold that contained growth factors BMP-2, VEGF, poly(lactic-co-glycolic acid) (PLGA), and mesenchymal cells. They used β-TCP as a component principle of calcium phosphate powder for the creation of the microspheres. They demonstrated that the scaffold composite was biocompatible and enhanced osteogenesis and angiogenesis in vitro. Then, using rabbits as an animal model, they observed that by means of radiographic and histological analysis the BMP-VEGF-PLGA-CPC scaffolds exhibited good biocompatibility, as well as osteogenic and angiogenic activity in vivo.

The findings of the studies discussed above are related to the diamond concept of bone consolidation of the biological chamber proposed by Calori *et al.* (124), where, in order to obtain adequate bone consolidation or regeneration, three fundamental elements must be present. These are 1) growth factors or signaling molecules (osteoinduction), 2) osteoprogenitor cells (osteogeneration), and 3) the extracellular matrix/natural scaffold (osteoconduction). Although we consider that this approach is the future of ONFH treatment in pre-collapse phases, our study did not include a treatment group with the three elements, since its objective was to demonstrate that treatment groups with bone marrow concentrate or BMP2 combined with β-TCP were superior to central decompression.

Since its approval by the Food and Drug Administration (FDA) in the USA, the use of BMP2 as a bone graft substitute has been common in orthopedics, especially in spine fusion surgery (245–247). Although it has proven to be an effective product, its use has been linked to adverse effects that have generated much controversy. It has been related to heterotopic ossification (248,249), inflammatory disorders (250), urogenital events (251), wound disorders (252), and tumor formation risk (253).

In our animal study, none of the sheep presented complications related to the surgical wound. This could also be ruled out by X-ray heterotopic ossification. Once the proximal femur was extracted for analysis, we were able to rule out any inflammatory process at the local level. One of the ONFH experimental studies where BMP2 (221) was also used as a treatment also reports no observed soft tissue ossification or foreign body reaction. The authors of this study used the same dose of BMP2 as used in our study: 300 µg of recombinant BMP-2. The dose seems to have a direct relationship with

the risk of presenting complications and does not necessarily increase its effectiveness in bone regeneration (254). In our animal study, we can confirm that the use of BMP2 for the treatment of ONFH is safe and is not related to any complications.

20. - Limitations

The main limitation of the present study is the number of sheep included in the groups. Initially, the idea was to include six sheep per group, sheep being considered large experimental animals. The Ethical Committee of Animal Experimentation (CEEA) of the Research Institute of the Vall d'Hebron University Hospital (VHIR) considered that it was an appropriate number and that it respected the principles of animal research. Ultimately, we managed to include only four per group due to the complications presented by the sheep during the course of the study. This prevented us from being able to make powerful conclusions in relation to the results obtained in our study.

A second limitation is the follow-up time after the treatment of ONFH. The sheep had to remain in the animal facilities of the Research Institute of the Vall d'Hebron University Hospital (VHIR) throughout the study due to current legal regulations, which forced us to limit the follow-up time to six weeks because of the animal housing limited physical capacity. This fact may have affected the results obtained in our study, so we recommend in a future study to follow up at least 12 weeks after the treatment of ONFH.

CONCLUSIONS

1. A new modified preclinical model of ONFH using minimally invasive administration of cryotherapy with helium-argon gases induces ONFH in pre-collapse phases in a safe, quantitative and reproducible way.

2. The use of core decompression isolated or associated with biomaterials stimulates bone regeneration in an animal model in sheep of ONFH.

3. A core decompression associated with bone marrow concentrate combined with β-TCP is not superior to isolated core decompression in a sheep animal model at six weeks of follow-up.

4. A core decompression associated with BMP-2 combined with β-TCP is not superior to isolated core decompression in a sheep animal model at six weeks of follow-up.

5. The use of biomaterials in our animal model of ONFH turned out to be a safe treatment without adverse reactions.

6. The results of our study do not support the routine use of BMP-2 or bone marrow concentrate associated with β-TCP granules in the treatment of pre-collapse ONFH.

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