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New preclinical porcine model of femoral head osteonecrosis to test mesenchymal stromal cell efficiency in regenerative medicine

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Abstract
Purpose In order to evaluate new therapeutic approaches to human osteonecrosis of the femoral head (ONFH), this study proposed to improve the existing animal model by developing a new surgically induced pig model.
Methods First, ONFH was induced with an easy and minimally invasive technique: cryogenic insult with repeated freeze–thaw cycle. Then, to compare and improve the efficacy of this first method, we combined the cryogenic insult to vascular coagulation of the posterior circumflex vessels.
Results Cryoinjury with repeated freeze–thaw cycle alone is sufficient to induce, three weeks postsurgery, a subchondral necrosis as confirmed by magnetic resonance imaging (MRI) and histological analysis. However, a bone regeneration began at four weeks and was complete at eight weeks. To optimise this result, we combined cryoinjury with posterior circumflex vessel coagulation and observed the persistence of ONFH, with progression to collapse at 14 weeks postinduction.
Conclusions Cryoinjury associated with partial vascular coagulation is sufficient to obtain localised and sustainable necrosis in the subchondral area of the femoral head, reproducing all stages of the human disorder. The co-analysis by MRI and histology allowed us to confirm that the classic T1- and T2-weighted hyposignal regeneration front around a fatty high T1-weighted signal observed by MRI indicate signs of induced osteonecrosis. Our results indicate that our pig model induces all stages of human ONFH, which can be followed by MRI, making it relevant for clinical trials.

Keywords Femoral head osteonecrosis · Preclinical porcine model · Regenerative medicine · Cryoinjury · MRI · Mesenchymal stromal cells

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Introduction

Osteonecrosis of the femoral head (ONFH) is a bone vascular disorder that occurs in young patients (mean age 36 years) [1]. This disease is often considered to be idiopathic; however, a great number of risk factors or aetiologies are listed, including corticosteroid administration, alcoholism and vascular disorder (e.g. sickle cell disease, endothelial nitric oxide synthase polymorphism), fat emboli and certain autoimmune diseases [2–4]. ONFH is due to the loss or compromise of blood flow to the femoral head (FH) and bone-progenitor deficiency, leading eventually to FH collapse [5]. Magnetic resonance

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imaging (MRI) provides early diagnosis and quantitative assessment, displaying the classic T1- and T2 -weighted hyposignal regeneration front around a fatty, high T1-weighted signal [6]. After collapse, hip replacement is the best therapeutic option [7]. However, in young patients, these options are often unsatisfactory [8].

To delay collapse, there are several treatment options in the early stage of ONFH, including core decompression associated or not to autologous bone marrow (BM) grafting. Core decompression reduces mechanical stresses and enhances bone repair, but reconstruction remains incomplete [9]. Decompression combined with autologous BM grafting is a more effective treatment option [10, 11]. BM contains several progenitor cells, such as mesenchymal stromal cells (MSCs), which play an important role in bone regeneration [12]. However, the success of this approach is limited to the early stage of the disease, probably due to the small number of MSCs in the concentrate BM (0.001–0.01 %) [10]. Several studies indicate that bone-tissue engineering using osteoprogenitor cells, such as MSCs, represents a promising therapeutic approach and provides an alternative to autologous BM grafting for treating bone defects [13–18].

In order to test the efficacy of MSCs in ONFH treatment, animal models are needed. Several small-animal models, including quadruped [19, 20] and biped models [21], were developed, but these models are not able to reliably reproduce all stages of the human disease; also, due to their small size, these animals are not suitable for diagnosing ONFH using MRI analysis. In contrast, large-animal models, such as emus or pigs, seem more appropriate, as their bone properties are similar to that of humans [22, 23]. To induce an ONFH in these large-animal models, a combination of ischemic and cryogenic insults were previously reported [24–26]. These techniques are highly invasive and lead to massive osteonecrosis associated with FH collapse and subcapital fractures. Bone freezing has been shown to induce osteoblast/osteocyte death, but to obtain vascular destruction, repeated freeze–thaw cycles are necessary [27].

The purpose of this study was to develop an animal model of ONFH by reproducing human pathology in order to evaluate different clinical approaches of bone regeneration. As the emu cannot be easily used in Europe, and because pig is commonly used to obtain preclinical data, we created a porcine ONFH model [23]. Vascular ligation is a highly invasive procedure causing serious complications postoperatively [22]. As repeated freeze–thaw cycles without vascular ligation has never been tested, we determined whether cryogenic insult with repeated freeze–thaw cycles was sufficient to obtain localised subchondral necrosis. We also evaluated the correlation between histological and MRI changes.

Materials and methods

Animals

Ethical approval for animal experimentation was obtained from the local ethical committee (File number: 12-036, notice number: 09/10/12-2). Eighteen female pigs (Landrace hybrid and large white pigs) weighing 35–50 kg and aged three to six months were studied (Lebeau Christian, Gambais, France). Pigs were managed following instructions by the ethics committee.

In vitro test of cryoinjury on porcine FH

Cryoinjury was achieved using a 4-mm Joule-Thompson cryoprobe (ERBE Medical, Limonest, France) (Fig. 1a). Medical nitrous oxide (Air Liquide, Paris, France) allowed the cryoprobe tip to freeze to −80 °C in a few seconds. After a 5 mm drilling the cryoprobe was placed in the centre of the porcine FH (n=6). Temperature sensors (Digi-Sense Dua Log R Thermocouple, Cole Parmer, Illkrich, France) were then positioned, and their distances from the probe tip were estimated after dissection of the FH (between 1 and 10 mm) (Fig. 1b, d). To reproduce the standard physiological conditions, the FH was placed in a 37 °C water bath (Fig. 1b). Nitrous oxide was delivered consistently for 15 minutes, and the temperature was measured every 30 seconds (Fig. 1c).

Surgical procedure

Protocol 1 (n=9) used a percutaneous approach to the hip. A 1-cm incision was made next to the greater trochanter. Under fluoroscopic guidance, a 5-mm tunnel was drilled along the femoral neck, through the growth plate and into the centre of the FH. The cryoprobe was then introduced using this tunnel, and its correct placement was checked by fluoroscopy (Fig. 2a). To induce the cryogen insult, the FH was exposed to two freezing cycles of 15 minutes, separated by spontaneous warming for ten minutes. Protocol 2 (n=9) was an open lateral approach through the gluteus maximus fibres to expose the lateral aspect of the greater trochanter. With specific rotation of the hip, the trochanteric pelvic muscles were exposed. The posterior circumflex vessels were coagulated with diathermy (Fig. 2b). Access to the FH and cryogen insult were done as for protocol 1 (Fig. 2c).

Magnetic resonance imaging (MRI)

MRIs were systematically performed using the 1.5-T device (Siemens Avanto, Erangen, Germany). Pigs were in supine position, with their hind legs tied in extension. T1-weighted images (T1) and T2-weighted images with fat saturation (T2 FS) were obtained in the coronal and axial planes. The lateral FH was used as control.

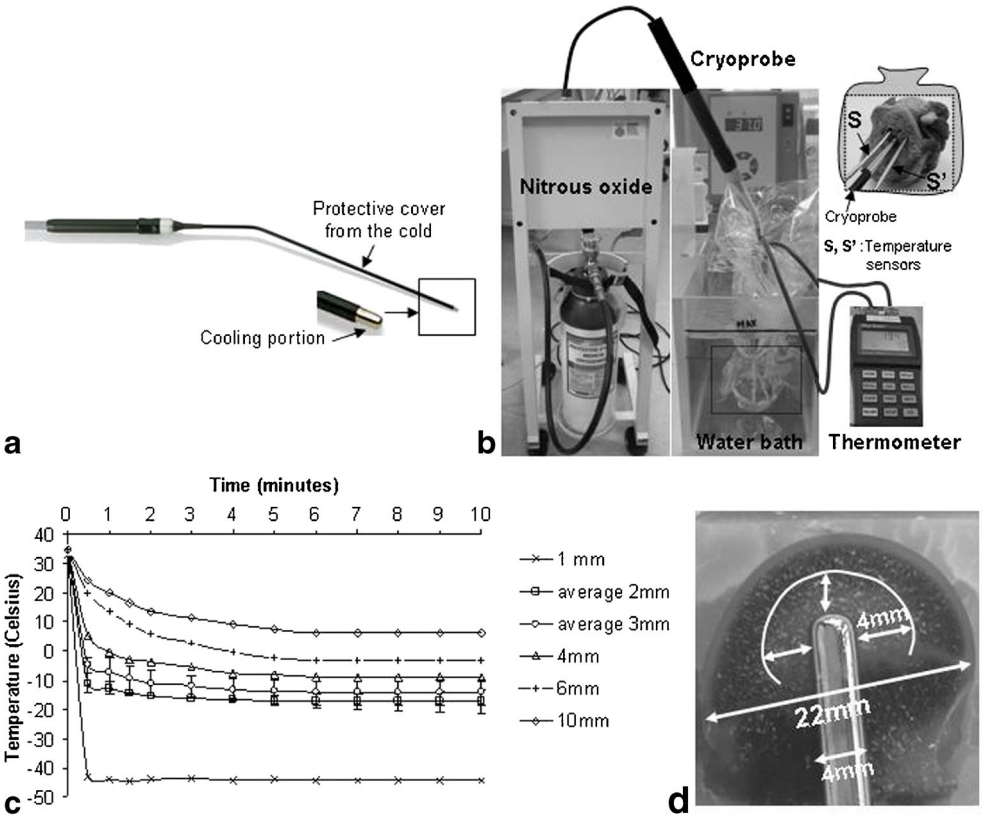


Fig. 1 In vitro test of cryoinjury of porcine femoral head (FH), **a** Cryoprobe and magnification of extremity. **b** In vitro protocol: FH is placed in a plastic bag at 37 °C in a water bath. The cryoprobe is positioned in the centre of the FH, and temperature sensors are placed on either side of the cryoprobe at distances varying between 1 and 10 mm. **c** Temperature during cryoinsult induced by a cryoprobe positioned at the center of the head (n=6). **d** Explanatory macroscopic image of an hemisection of the FH, illustrating the cryoprobe position and the radius of cold diffusion

Histology

After animal sacrifice, FHs were fixed with 4 % formaldehyde solution (VWR, Fontenay Sous Bois, France). The lateral FH was used as control. After decalcification with 6.8 % nitric acid (VWR) for two weeks, the samples were rinsed in tap water and embedded in paraffin. Sections (3 μm) were stained with Masson's trichrome.

Results

In vitro test of cryoinjury on porcine FH

In vitro experiments of cryoinjury showed a rapid but localised spread of cold in bone tissue (n=6 FH). After 30 seconds of cryoinsult, sensors placed between 0.5 and 1 mm distance from the cryoprobe measured a temperature of −45 °C. The temperature increased with the distance to the probe, with −18 °C at 3 mm and between −9 °C and −3 °C at around 5 mm. The temperature became positive at 10 mm, with a measure of + 6 °C (Fig. 1c). Interestingly, the temperature stayed localised and constant over time (15 minutes).

In vivo test: protocol 1

As the spread of frost was constant over time, and because Gage et al. showed that combining repeated freeze–thaw cycles enhances cell death, we decided to create a three-step protocol with two cryoinjuries of 15 minutes separated by a ten minutes thaw for in vivo osteonecrosis induction [27].

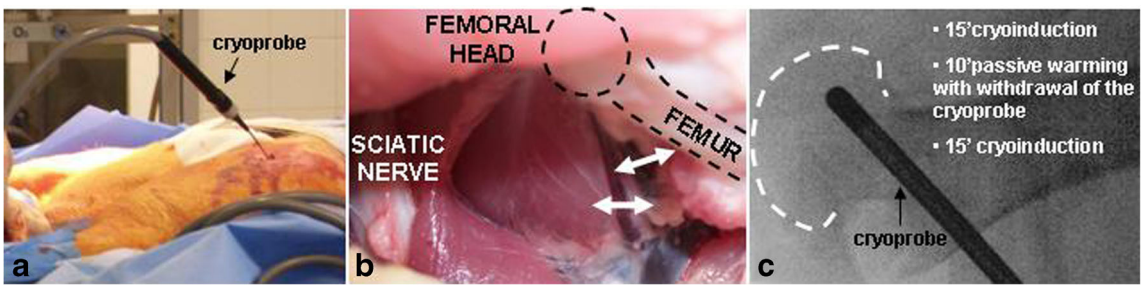


Fig. 2 Surgical procedure. **a** Percutaneous approach of protocol 1 with 2 cycles of freezing/thawing induced by a cryoprobe. **b** Step 1 of protocol 2, with coagulated vessels close to the femoral head (FH). **c** Fluoroscopic image of FH with cryoprobe

After this protocol, all pigs were able to bear full weight after a maximum of 48 hours.

Histological findings

Porcine control FH were normally constituted of a regular trabecular bone network containing osteocytes (Fig. 3a, c). Trabeculae were surrounded by BM endowed with haematopoietic cells and adipocytes (Fig. 3b). As our pigs were young, a growth plate was still present in the middle of the FH (Fig. 3a).

Two weeks after cryoinjury, around the drill hole we observed a 4-mm necrotic area in subchondral bone (n=3) (Fig. 3d). In the necrotic zone, osteocyte lacunae were empty, indicating osteocyte death (Fig. 3e). The haematopoietic islands were replaced by a dense fibrous tissue and by characteristic necrotic adipocytes (i.e. adipocytes characterised by large vacuoles with absence of nuclei and rupture of cells walls) (Fig. 3e, f). These characteristics were consistent with early stage of osteonecrosis [26]. After four weeks, perforation trace and necrotic areas were still present (n=4) (Fig. 3k). However, the fibrotic marrow and necrotic adipocytes were less dense than two weeks after surgery, and few haematopoietic cells were observed (Fig. 3l). Signs of early stages of bone regeneration, characterised by lining osteoblasts producing immature osteoid bone matrix and osteocytes in lacunae of the new bone, were also observed (Fig. 3l). After eight weeks, normal bone with new BM elements was observed (Fig. 3n). Only a small scar of fibrosis was still noticeable (n=2) (Fig. 3m, o).

MRI results

Three weeks after cryoinjury, coronal MRI was analysed (n=1) (Fig. 3g–j). We observed the classic MRI appearance of osteonecrosis with a front of regeneration: a geographical region of decreased marrow signal within the normally bright fat of the FH on T1-weighted images, and mixed low/high signal on T2 FS (Fig. 3h, j). The front of regeneration delimitated a fatty necrotic area in the epiphysis of high T1 and T2 FS signal (Fig. 3h, j). We noticed drilling rearrangement, aspecifically of high T2 FS and low T1 signal (Fig. 3h, j). These results were compared with the control (right) FH (Fig. 3g, i).

In vivo test: protocol 2

As bone regeneration was observed four weeks after cryoinjury, we decided to induce a more profound vascular insult by adding coagulation of the posterior circumflex vessels. After surgery (protocol 2), all pigs were able to bear full weight after a maximum of 48 hours.

Fig. 3 Repeated freeze–thaw cycles with cryoprobe, protocol 1. **a**–**f**, **k**–**o** Histological analysis of porcine femoral head (FH) stained with Masson's trichrome. **a**–**c** Normal FH; **d**–**f** FH 2 weeks after cryoinjury; **k**, **l** FH 4 weeks after cryoinjury; **m**–**o** FH 8 weeks after cryoinjury. **a**, **d**, **k**, **m** Magnification ×1; **a** *black arrows* indicate the growth plate, **d**, **k** necrotic area and cryoprobe hole are defined by a *continuous* and *dashed line*, respectively; **m** area fibrosis, *small* and *large dashed lines*; **b**, **c**, **e**, **f**, **l**, **n**, **o** magnification ×20. *Hc* haematopoietic cells, *BM* bone marrow, *A* adipocytes, *Ob* osteoblasts, *Oc* osteocytes, *TB* trabecular bone, *NO* necrotic osteocyte, *NA* necrotic adipocyte, *FT* fibrous tissue, *NB* new bone. **l** *Large dashed lines* define the zone between NB and NO. **g**–**j** Coronal magnetic resonance imaging (MRI) 3 weeks after cryoinjury: *blue arrows* indicate regeneration front, *red arrows* indicate fatty necrosis area, *green dashed lines* surround the drilling rearrangement. **g** T1-weighted image of control (right) FH; **h** T1-weighted image of the left FH 3 weeks after cryoinjury (test FH); **i** T2-weighted image of the control (right) FH; **j** T2-weighted image of the left FH 3 weeks after cryoinjury (test FH)

MRI results

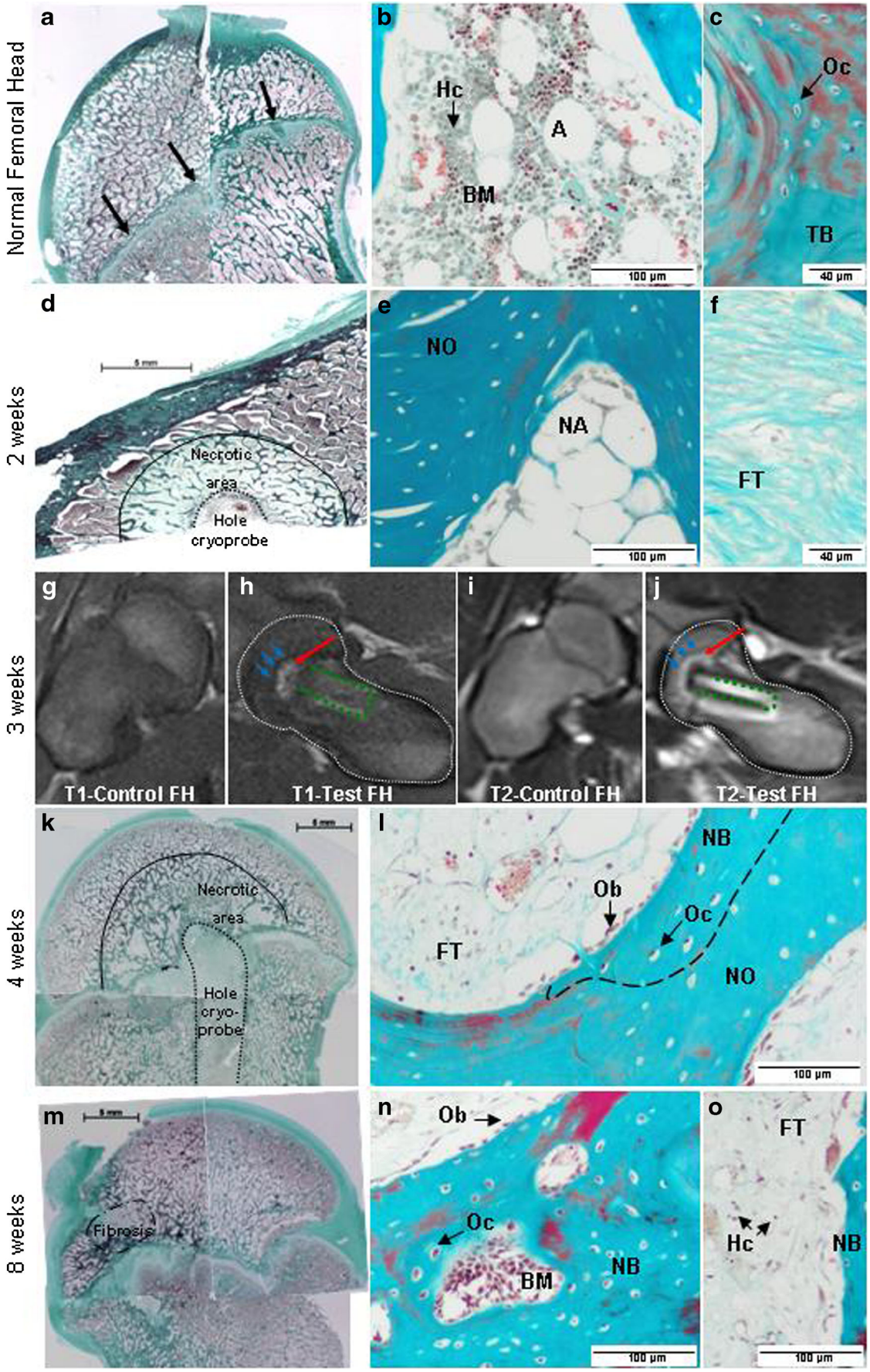
At two (n=1), 3 (n=5) and four (n=2) weeks after protocol 2, MRI images displayed a fatty necrotic area of high T1 and T2 FS signal within nonspecific high T2 FS and low T1 oedema on six of eight FH (Fig. 4a, b, Table 1). We also noticed a drilling rearrangement, specifically of high T2 FS and low T1 signal. At six (n=1) and eight (n=2) weeks after insult, we observed the classic MRI appearance of osteonecrosis with a stable necrotic area of high T1 and T2 signal and a front of regeneration on all FH (Fig. 4e, f, Table 1). Fourteen weeks after surgery (n=2), coronal T1 and T2 images showed head collapse. Drilling areas were back to normal, with homogeneous fatty bone medullary signal (Fig. 4i, j).

Histological finding

For each MRI image with necrosis characteristics, histological analysis was performed on at least one FH (Table 1). At two and three weeks after protocol 2, histological analysis indicated the presence of necrotic area (4–5 mm) around the drill hole (Fig. 4c, Table 1). A similar necrotic profile was maintained at four and six weeks (Fig. 4g, Table 1). All histological features of necrosis were observed in the subchondral area, at the edge of the growth plate (i.e. empty lacunae; fibrous tissue; necrotic adipocyte; necrotic osteocyte) (Fig. 4d, h). At 14 weeks, histological examination revealed a loss of sphericity of the articular surface, with collapse of subchondral bone and persistence of osteonecrotic changes (Fig. 4k, l).

Discussion

This study proposes a new, large-animal (porcine) model of ONFH that is able to reproduce the different stages of human osteonecrosis from early MRI signal changes to collapse of


Normal Femoral Head
a
b
Hc
A
BM
100 μm
c
Oc
TB
40 μm
2 weeks
d
5 mm
Necrotic area
Hole cryoprobe
e
NO
NA
100 μm
f
FT
40 μm
3 weeks
g
T1-Control FH
h
T1-Test FH
i
T2-Control FH
j
T2-Test FH
4 weeks
k
5 mm
Necrotic area
Hole cryo-probe
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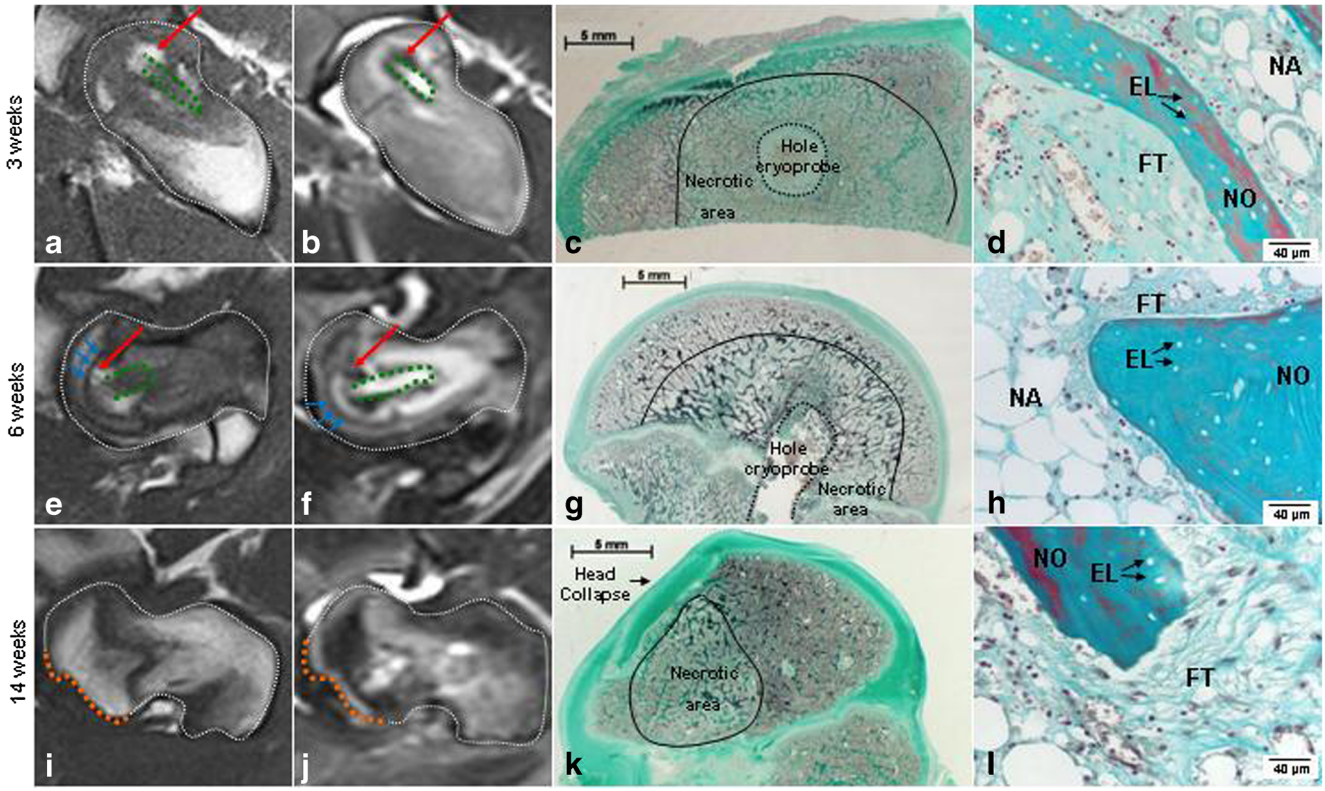

Fig. 4 Cryoinjury combined with partial vascular coagulation, protocol 2. Analysis of the cryoinjured femoral head (FH): **a**–**d** at 3 weeks, **e**–**h** at 6 weeks, **i**–**l** at 14 weeks. **a**, **b**, **e**, **f**, **i**, **j** Coronal magnetic resonance image (MRI) analysis: *blue arrows* indicate the front of regeneration, *red arrows* indicate the fatty necrosis area, *green dashed lines* surround the drilling rearrangement and *orange dashed lines* indicate FH collapse. **a**, **e**, **i** T1-weighted image; **b**, **f**, **j** T2-weighted image with fat saturation. **c**, **d**, **g**, **h**, **k**, **l** Histological analysis: **c**, **g**, **k** magnification ×1; **d**, **h**, **l** magnification ×20. *B* bone, *BM* bone marrow, *Oc* osteocytes, *EL* empty lacunae, *NO* necrotic osteocyte, *NA* necrotic adipocyte, *FT* fibrous tissue. Necrotic area, *solid line* and hole cryoprobe, *small dashed lines*

the FH in the final stage. Cryosurgery using a repeated freeze–thaw cycle was sufficient to damage the vascular system (−20 °C), and to be lethal to osteocytes (−2 °C) [27]. Moreover, holding a constant temperature had substantial impact on the volume of the induced necrotic lesion [28]. Our in vitro study indicates rapid and good spread of cold through bone tissue, associated with a constant temperature. These results demonstrate that the cryoprobe seems to be sufficient to obtain localised subchondral necrosis representing half of the FH, which is consistent with what is observed in human ONFH [4]. Our in vivo results confirm that two freeze–thaw cycles systematically induce necrosis at three weeks by destroying bone cells and proximal vessels [27]. However, this method is not sufficient to obtain a durable ONFH, as bone progenitor cells are able to colonise the necrotic area as soon as four weeks. This may be due to the presence of the growth plate, which is a reservoir of progenitor cells.

In order to achieve sustainable and reproducible ONFH mimicking human disease, we combined cryoinjury with a partial vascular coagulation of posterior circumflex vessels. Following this protocol, we reproduced all stages of human ONFH, with segmental collapse after 14 weeks, as observed on MRI scans and confirmed on histological examination. These results confirm the importance of destroying the vascular network around the FH to obtain an evolutive ONFH [24]. Recently a sheep model of ONFH combining total vascular ligation of all circumflex vessels, hip luxation and cryoinjury

Table 1 Protocol 2: magnetic resonance imaging (MRI) and histology analysis

FH Number	Delay to imaging	MRI characteristics of necrosis	Histological characteristics of necrosis at sacrifice
1	2W	Y	Y
2	3W	Y	Y
3	3W	Y	Y
4	3W	N	N
5	4W	Y	Y
6	4W	N	N
7	6W	Y	Y
8	3W	Y	
	8W	Y	
	14W	Y	Y
9	3W	Y	
	8W	Y	
	14W	Y	Y

MRI magnetic resonnance imaging, *FH* femoral head, *W* weeks, *Y* yes, *N* no

was developed [26]. Although that technique mimics all stages of ONFH, it also causes cartilage damage, which is not a hallmark of human ONFH. Our results indicate that vascular coagulation of the posterior circumflex vessels combined with cryoinjury is sufficient to induce ONFH and that there is no need to associate coagulation of the anterior circumflex vessels and/or dislocate the hip to obtain the same result.

Pigs exhibit a physiological behaviour similar to humans, and in contrast to sheep, pigs present biomechanical properties, bone mineral content and density close to those of human bone [23]. However, pigs have an immature skeleton until approximately the age of six years, and for technical and housing reasons, it is not possible to manage experiments after this age [29]. Therefore, one of the main limitations of our pig model is the presence of the growth plate. This may explain the spontaneous bone regeneration we observed after simple cryoinjury (protocol 1) and why two pigs failed in protocol 2.

The classic MRI features of osteonecrosis are always associated with a histological necrosis, indicating good concordance between histological and MRI changes. The ability to monitor osteonecrosis by repeated MRI scans makes the pig model relevant for clinical trials. The effectiveness of conservative treatment is thought to be better when performed in the early stage of ONFH [10]. Future studies will analyse treatment/response in animals in early stages of ONFH. In our model, we observed early-stage necrosis between three and six weeks after cryoinsult with progression to collapse at 14 weeks. Therefore, in the first month following necrosis induction, this model can be used to test innovative conservative treatments for ONFH, such as injection of amplified MSCs. Treatment outcome will first be monitored by changes seen on MRI and the occurrence of collapse, and then confirmed by histology.

Conclusion

Cryoinjury associated with partial vascular coagulation is sufficient to obtain localised and sustainable subchondral necrosis of the FH, reproducing all stages of the human disease. Moreover, the good correlation between histological and MRI results indicates that the pig is a relevant model for future ONFH clinical trials.

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