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Emu Model of Full-range Femoral Head Osteonecrosis Induced Focally by an Alternating Freezing and Heating Insult

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The emu, a large bipedal bird with hip joint biomechanics similar to humans, was used to establish an experimental model of femoral head osteonecrosis and subsequent femoral head collapse. Focal lesions were induced in 20 adult male emus using an alternating liquid nitrogen freezing and radiofrequency heating insult. At 2, 4, 8, 12 and 16 weeks post-surgery, hip magnetic resonance imaging (MRI) was performed. Before the emus were sacrificed, barium sulphate was infused to the lower extremity to study blood vessel distribution patterns. Femoral samples were scanned by micro-computed tomography (micro-CT) and evaluated histologically. Hip MRI showed changes from broad oedema to femoral head collapse. Emus developed a crippled gait from post-operative week 6. Micro-CT scans and histology showed human-like osteonecrotic changes with an impaired local blood supply. The protocol resulted in consistent full-range osteonecrosis of the femoral head that may serve as a model for testing potential treatments.

KEY WORDS: OSTEONECROSIS; FEMORAL HEAD; BIPEDAL ANIMAL MODEL; EMU; MAGNETIC RESONANCE IMAGING; MICRO-COMPUTED TOMOGRAPHY; HISTOLOGY

Introduction

Osteonecrosis of the femoral head (ONFH) is a common orthopaedic disorder, with an incidence of between 10,000 and 20,000 per year in the USA.1 ONFH usually occurs in people in their late 30s and early 40s.1 Untreated, osteonecrosis generally progresses to femoral head collapse, and subsequently to secondary osteoarthritis.1,2 Total hip replacement for osteonecrosis is associated with an early loosening rate of 10%3 and in the long term, the rate is even higher.4 Although many methods to preserve the femoral head have been proposed, few have been found, to a certain extent, to be satisfactory.5,6 Consensus as to which is the best treatment is, however, lacking. This controversy is due, in part, to a lack of ability
to compare treatments systematically using an animal model with a natural history similar to that of human femoral head collapse.\textsuperscript{7}

Quadrupedal animal models, such as rabbits, have been intensively used for studying the pathophysiology of osteonecrosis,\textsuperscript{8,9} although without resulting in joint collapse due to ONFH. Previously, an animal model of ONFH using emus was proposed.\textsuperscript{10} Emus are peaceful, ostrich-like, large birds that weigh approximately 50 kg, are 1.5 m tall and reach skeletal maturity at 1 year of age.\textsuperscript{10} In a previous emu model, surgically-induced necrotic lesions were found to progress consistently to a human-like structural collapse of the hip joint.\textsuperscript{10} Necrosis in this earlier study was achieved 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.\textsuperscript{10} This model could, however, still be improved in several respects, such as reducing sub-capital fractures due to the freezing insult, reducing the lesion size to more human-like, segmental type lesions and quantifying the liquid nitrogen administration and the intracephalic temperature.\textsuperscript{10,11} To permit control of the key necrotic lesion parameters, a cryo-insult instrument with internal closed circulation of liquid nitrogen was developed.\textsuperscript{11} Besides precluding direct contact of the liquid nitrogen with the tissue bed, closed circulation has the important advantage of avoiding spurious occlusion of the liquid nitrogen flowstream because of tissue-fluid ice formation.\textsuperscript{11} The instrument successfully produced segmental lesions in emus, but did not result in femoral head collapse.\textsuperscript{11}

Many reports have demonstrated that osteonecrosis can be induced by radiofrequency (RF) treatment.\textsuperscript{12–16} The idea of combining freezing and heating was first proposed in 1982.\textsuperscript{17} A previous study demonstrated that alternating liquid nitrogen freezing and RF heating substantially improved the treatment of soft-tissue tumours, in a manner not achievable by freezing or heating alone.\textsuperscript{18,19} We postulated that using an alternating freezing and heating insult could improve osteonecrosis induction and subsequent joint collapse. Accordingly, the aim of the present study was to: (i) improve the existing animal model of ONFH; and (ii) confirm the postulation that effective induction of ONFH with hip-joint collapse could be achieved with a focal alternating liquid nitrogen freezing and RF heating insult.

### Materials and methods

#### EMUS

Twenty adult male emus were used. The Animal Care and Use Committee of Chinese People’s Liberation Army General Hospital approved the experimental protocol (permit number 2010-0209). The Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) were followed, as were Chinese laws on animal protection, where applicable. Emus were maintained on a 12-h light–dark cycle and received food and water ad libitum.

#### INSTRUMENT USED TO CREATE ONFH

The alternating freezing and heating system, designed by Shanghai Jiao Tong University, consists of 13 parts (Fig. 1A). Liquid nitrogen flows out of the Dewar and into the cryoheating probe, where the fluid circulates within the probe to generate the freezing effect. The cryoheating probe is also a simultaneous RF emission electrode; its structure is illustrated in Fig. 1B. This probe...
has three parts: the 3-cm long active part; the vacuum insulation part that protects normal tissue; and a handle. An inner tube of 1 mm in diameter is placed inside the probe, through which nitrogen flows in a closed circuit. The diameters of the heat-transfer section and the vacuum-insulation section are 2.5 mm and 5 mm, respectively. This probe can be cooled to –150 °C in approximately 2 min, which ensures rapid freezing in the first step of treatment.\textsuperscript{18,19} For the subsequent rapid thawing and heating processes, RF was chosen to achieve deep volumetric heating through longer electromagnetic waves. With this system, the freezing or heating capacity of the probe can be precisely regulated by controlling the flow of nitrogen and the RF power.\textsuperscript{18,19}

**SURGICAL PROCEDURE**

Surgery was performed under sterile conditions. Emus were fasted for 12 h before surgery, then received 0.02 mg/kg atropine and 50 kIU/kg penicillin, then after 30 min, 30 mg/kg ketamine hydrochloride (Alfasan, Woerden, The Netherlands) with 10 mg/kg xyazine (Alfasan); all drugs were given by intramuscular injection. Once emus were sedated, a catheter was placed in the external jugular vein and anaesthesia was maintained intravenously using 6 mg/kg per h ketamine hydrochloride. The emus were placed in a left lateral position. The region of the right hip was sheared and the skin was cleaned with soap and disinfected with iodine solution. Then, the right greater trochanter was approached by a direct lateral incision. A guide instrument was constructed to position the tip of the cryoheating probe juxta-articularly and at angles 60° superior and 0° medial relative to the femoral shaft. The positioning accuracy of the guide instrument was tested in both denuded intact cadaveric femora of emus.
and in situ in intact animal cadavers (Fig. 2). Then the instrument was used to create osteonecrosis by a focused alternating freezing and heating insult. To compare the model with the previous emu model of ONFH, the same freezing setting was used. The programme was 9 min of liquid nitrogen freezing followed by 5 min of RF heat as the active thaw, repeated twice. The heat insult was controlled at a 50 °C holding temperature around the probe. The probe and guide instrument were removed and the cortical hole was sealed with bone wax. The surgical field was irrigated with warm saline solution and the incision was closed in anatomical layers. After the emus recovered, they were free to roam and eat.

POST-SURGICAL PROTOCOL
The emus’ gaits were recorded by observation every 2 days. Emus underwent sequential magnetic resonance imaging (MRI) examinations at 2, 4, 8, 12 and 16 weeks post-surgery to study the progression of osteonecrosis. To compare the incidence of osteonecrosis at different time points, three emus were chosen randomly and sacrificed at week 12. The remaining emus (n = 17) were sacrificed at week 16. Before the emus were sacrificed, barium sulphate was infused into the lower extremity for studying patterns of blood vessel distribution as specified below. Under general anaesthesia, a midline incision was made to expose the abdominal aorta and the inferior vena cava. A cannula was distally inserted into the vessels and proximal vessels were ligated. The cannula of the abdominal aorta was used to inject 500 ml of heparinized saline (25 000 IU in 0.9% sodium chloride) followed by continuous irrigation with a natural saline solution; the liquid flowed freely through the inferior vena cava. After the residual intraosseous blood was washed out, 3000 ml of 10% formalin was injected slowly via the abdominal aorta, and then 3000 ml of 15% barium sulphate suspension in 4% glutin solution (G1890; Sigma-Aldrich, St Louis, MO, USA) was infused quickly. The sample was placed at 4 °C for 4 h to allow the glutin solution to solidify. Next, the bilateral femora (treated and untreated control) were excised and fixed in a 10% buffered neutral formalin solution.

The specimens were scanned with microcomputed tomography (micro-CT) (RS-...
9, GE Healthcare, Ontario, Canada) at 46 µm resolution before being placed in 25% formic acid solution for decalcification. Images were reconstructed and data were analysed using the built-in software of the GE micro-CT equipment. The same volume of bone was chosen from the corresponding region of the femoral head for both the contralateral control specimens and the operated sides. Then, the maximum intensity projection and isosurface were used to visualize the vessels in the proximal femur region. The femoral head was divided into three zones, similar to those used for regional evaluation of ONFH in patients: the reparative, necrotic and intact zones, according to vessel distribution. The microstructure of the trabecular bone and the Hounsfield Units (HU) in the different zones were evaluated. After decalcification, the samples were cut in half longitudinally and embedded in paraffin. Central sections of 5 µm in thickness from the femoral head were cut with a Leitz 1512 microtome (Leica, Wetzlar, Germany) and stained with haematoxylin and eosin (H&E) for histological examination.

**STATISTICAL ANALYSES**
Statistical analyses were carried out using the SPSS® statistical package, version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows®. The incidence of femoral collapse – defined as the number of emus in which the femoral head collapsed, divided by the total number of emus, at week 12 and week 16 – was compared using the χ²-test. The volume of collapse was defined as 100% minus the volume of the collapsed femoral head, divided by the volume of the contralateral intact femoral head. The comparison between weeks 12 and 16 was performed using an unpaired t-test. A P-value of < 0.05 was considered to be statistically significant.

**Results**

**GAIT CHANGES**
All 20 emus walked without assistance within 3 h after surgery. No animal demonstrated signs of infection or crippled gait in the first 6 weeks post-surgery. Crippled gait developed in 14/20 (70%) emus at week 10 after surgery, then in all animals at week 16. The symptoms began with subtle limping for 1 – 2 weeks, then a worsening to an obvious crippled gait for 1 – 3 weeks, and resulted in recumbency in some emus.

**MRI RESULTS**
The MRI scans of all emus before week 4 post-operatively showed broad oedema on the femur, including the femoral head, shaft and condyle (Fig. 3A). The oedema observed on MRI images reduced gradually with time; shrinking to the proximal part of the femoral head at week 8 (Fig. 3B). The oedema extended to all regions of the femur in only two emus. At week 12, some emus showed obvious signs of femoral head collapse, accompanied by arthritic changes including joint effusion, variation in cartilage thickness and subchondral bone injury (Fig. 3C). One emu showed femoral head collapse and hemidislocation of the hip at week 12 (Fig. 3D). Some emus showed the human-like double-line sign at the femoral head, at week 8 (Fig. 3E).

**MICRO-CT RESULTS**
Micro-CT images showed both trabecular bone structure and vessel distribution as evidenced with perfused contrast medium. Comparison of the trabecular bone and vessels in the femoral head among specimens at different stages is shown in Fig. 4. The trabecular separation of the reparative zone was smaller than that of the intact zone. HU of the necrotic zone was similar to that of the intact zone, but smaller
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The vessel distribution or its density was improved remarkably at the proximal region of the collapsed femur, compared with the control. The contour altered in collapsed femoral heads, with obvious segmental collapse apart from flattening (Fig. 5) that mimicked joint collapse in human ONFH.

Two out of three emus (66.7%) showed femoral head collapse at week 12. At week 16 after surgery, 14/17 (82.4%) emus showed signs of femoral head collapse. There was no significant difference found in the incidence of femoral head collapse between weeks 12 and 16. The volume of collapse was 16.34 ± 8.11% (range 10.61 – 22.08%) at week 12 compared with 12.96 ± 6.74% (range 5.24 – 27.53%) at week 16; these findings were not significantly different.

HISTOLOGICAL FINDINGS
Histological examination revealed intact trabecular bone and normal marrow in the intact zone of the femoral head (Fig. 6A). In the necrotic zone, the normal marrow was replaced with fibrotic marrow and osteocytes had undergone cell death as shown by the

FIGURE 3: T1- and T2-weighted coronal magnetic resonance images (MRI) of representative emu femurs: (A) bilateral MRI of emu No. 19 at week 4, with right operated side (R) showing broad oedema and left control side (L) being intact (arrows show femoral head); (B) at week 8, operated side of emu No.19 showed significantly decreased oedema (arrows show femoral head); (C) at week 12, operated side of emu No.19 exhibited femoral head collapse (arrows show femoral head); (D) at week 12, operated side of emu No. 9 showed femoral head collapse and hemidislocation of the hip (arrow); (E) at week 8, operated side of emu No. 1 showed the human-like double-line sign (arrow)
FIGURE 4: Representative micro-computed tomography (micro-CT) images of collapsed femoral heads and contralateral intact controls in emus: (A) coronal section view at 46 µm resolution with trabecular bone fracture (red arrows); (B) the same coronal section view after transformation into Hounsfield Units (HU) – the two images used the same colour bar (colour changed from purple at 600 HU to red at 3000 HU); (C) three-dimensional reconstruction of the femoral head shown in Fig. 4A; (D) maximum intensity projection of the proximal femoral region (two-dimensional micro-CT-based angiography) – white shows the blood vessels; (E) three-dimensional micro-CT-based angiography reconstruction from Fig. 4D
empty lacunae (Fig. 6B). At the reparative interface, the destructive repair process was demonstrated and characterized by granulation tissue formation and creep toward the necrotic bone resorption region (Fig. 6C). In total, 15/20 (75%) emus showed empty lacunae (Fig. 6B). At the reparative interface, the destructive repair process was demonstrated and characterized by granulation tissue formation and creep toward the necrotic bone resorption region (Fig. 6C). In total, 15/20 (75%) emus showed...
juxta-articular fragmentation and segmental collapse with loss of contour of the articular surface, excluding one that experienced a full femoral head collapse.

Discussion

The bipedal emu was used to establish an experimental model of femoral head osteonecrosis that was able to mimic human mechanical failure of the hip joint, i.e. femoral head collapse, with good consistency. The model was established surgically by inducing lesions focally, by applying an alternating liquid nitrogen freezing and RF heating insult. The findings demonstrated that this alternating freezing/heating insult might induce significant bone cell and vessel destruction, and that the destructive–reparative process that developed in response to osteonecrosis might be the primary cause of the collapse of the necrotic femoral head.

The emu is an unconventional animal model in musculoskeletal research; it was chosen because it is a sizeable biped with hip-joint biomechanics similar to those of humans.7,21 Mature emus typically stand 1.5 m tall and weigh approximately 50 kg. On average, the emu takes 9563 steps/day, which extrapolates to 1.8 million hip loadings per year – a value that falls in the same general range as that seen in normal adult humans.21 As with the human walking gait, the emu ground reaction force shows a bimodal distribution over the course of the stance phase. The contact stress propensity of the proximal femoral head is generally similar to that of humans: the resulting hip contact force is of a similar relative magnitude (about 3.5 body weight maximum), direction (pointing distally along the axis of the femur), and location (on the anteroproximal surface of the femoral head).7,22

In the present study, histological examination of collapsed femoral heads revealed end-stage osteonecrosis with segmental collapse, as is observed in human osteonecrosis.23 Destructive repair leading to femoral head deterioration has also been found in quadruped models, but without resulting in femoral head collapse.8,9 Loading onto the osteonecrotic zone also contributes to femoral head collapse,24 and the differences in the hip-joint biomechanics between bipedal and quadrupedal animals might be the crucial factor resulting, or not resulting, in subsequent joint collapse.

Cryogenic and thermal insults have been increasingly adopted as laboratory vehicles for inducing bone necrosis. Although not among the causes of naturally occurring bone lesions, they are useful research tools because the main aspects of the resulting repair response reasonably replicate those of naturally occurring lesions.10,11,25,26 In cryosurgery, the freezing process causes direct cellular and vascular injury.27 Among the high-temperature treatment methods, RF is effective in treating many types of tumours.28 – 30 With hyperthermia treatment between 43 °C and 45 °C, the tissue temperature being moderately raised for a certain period of time is expected to induce cell death by affecting membrane fluidity and the cytoskeletal, protein and nuclear structures, and also disrupting DNA replication.31 Several reports have revealed that osteonecrosis can be induced by RF.12– 16 The idea of combining freezing and heating was first proposed by Gage et al.17 in 1982. Later, Hines-Peralta et al.32 proposed a hybrid device to enhance the efficiency of a bipolar RF system by placing a freezing unit between the two RF poles. Liu et al.33 designed a cryo-probe system with vapour heating, and found that freezing –
immediately followed by a rapid heating of the target tissue – could improve the treatment effect, due to thermal stress. A previous study demonstrated that an alternating liquid nitrogen freezing and RF heating insult – delivered with the system used in the present study to induce osteonecrosis of the emu femoral head – substantially improved treatment of soft-tissue tumours compared with rates achieved by freezing or heating alone.\textsuperscript{18,19} No previous studies have reported an animal model of ONFH induced by an RF-based insult or an alternating freezing and heating insult.

As the risk of joint collapse depends chiefly on the initial size and location of the necrotic lesion,\textsuperscript{34} a programmable alternating freezing and heating system was used to control the osteonecrotic range, and a guide instrument was used to control the location of the osteonecrotic lesion created within the femoral head of emus. This improved the consistency and incidence of femoral head collapse over previous emu models.\textsuperscript{10,11} The same duration of liquid nitrogen freezing, a similar freezing instrument and the same type of animal as used in the model of Reed \textit{et al.}\textsuperscript{11} were used, and this did not result in femoral head collapse. It could be argued that RF was able to induce osteonecrosis in animal models, and that the alternating liquid nitrogen freezing and RF heating insult might improve the bone-cell and vessel destruction. This system might be used in bone tumour treatment in the future.

Micro-CT images showed segmental collapse in the femoral heads of emus. According to microangiography, collapsed femoral heads showed an avascular zone and the density of vessels around this zone was higher than that in the intact zone. The segmental collapse observed in the bipedal emu model might also be explained by consistent reparative reactions around (or next to) the osteonecrotic region. In terms of the volume of joint collapse, the \textit{ex vivo} micro-CT data from the present study did not show a significant difference between the emus evaluated at weeks 12 and 16. This may be explained by the following two factors: (i) individual variations in the time to onset of joint collapse; and (ii) the technical limitation, in that we were unable to perform \textit{in vivo} micro-CT monitoring of joint collapse for this large animal. \textit{In vivo} micro-CT monitoring would be able to demonstrate the progression of joint collapse over time.

In conclusion, an alternating freezing and heating protocol was able to induce full-range ONFH in a bipedal emu model – with subsequent femoral head collapse that was similar to that observed in patients with ONFH – with excellent consistency. The establishment of this highly repeatable ONFH model with femoral head collapse could be used to evaluate potential treatments for the prevention of osteonecrosis-induced hip joint collapse in humans.

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\textbf{Conflicts of interest}
The authors had no conflicts of interest to declare in relation to this article.

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