RESEARCH

Impaired bone healing in rabbits with steroid-induced osteonecrosis

Corticosteroids are prescribed for the treatment of many medical conditions and their adverse effects on bone, including steroid-associated osteoporosis and osteonecrosis, are well documented. Core decompression is performed to treat osteonecrosis, but the results are variable. As steroids may affect bone turnover, this study was designed to investigate bone healing within a bone tunnel after core decompression in an experimental model of steroid-associated osteonecrosis. A total of five 28-week-old New Zealand rabbits were used to establish a model of steroid-induced osteonecrosis and another five rabbits served as controls. Two weeks after the induction of osteonecrosis, core decompression was performed by creating a bone tunnel 3 mm in diameter in both distal femora of each rabbit in both the experimental osteonecrosis and control groups. An in vivo micro-CT scanner was used to monitor healing within the bone tunnel at four, eight and 12 weeks postoperatively. At week 12, the animals were killed for histological and biomechanical analysis.

In the osteonecrosis group all measurements of bone healing and maturation were lower compared with the control group. Impaired osteogenesis and remodelling within the bone tunnel was demonstrated in the steroid-induced osteonecrosis, accompanied by inferior mechanical properties of the bone.

We have confirmed impaired bone healing in a model of bone defects in rabbits with pulsed administration of corticosteroids. This finding may be important in the development of strategies for treatment to improve the prognosis of fracture healing or the repair of bone defects in patients receiving steroid treatment.

Corticosteroids may be prescribed for several orthopaedic conditions, such as acute spinal cord injury and sciatica, but are more frequently used for the treatment of other medical conditions, including systemic lupus erythematosus, organ transplantation, rheumatoid arthritis and acute respiratory syndrome. Steroid-associated osteoporosis and osteonecrosis are common consequences that increase the risk of fractures. The pathophysiology of steroid-associated osteonecrosis and osteoporosis have been extensively studied. Histopathologically, steroid-associated osteonecrosis is the process of bone death with an inadequate repair response that frequently leads to subchondral collapse. Core decompression is often used in the treatment of early-stage osteonecrosis to remove necrotic bone, facilitate bone healing and prevent later joint collapse.

Only a few studies have reported adverse effects of corticosteroids on fracture healing or the repair of bone defects in the diaphysis of long bones. Several experimental models have been developed to study fracture healing in long bones such as the tibia in rabbits and the femur in rats or mice.

One of the most widely used models is a drill-hole defect at the metaphyseal region of a long bone. This has the advantages of bone healing without being influenced by the mechanical instability seen in fracture repair using external or internal fixation. This model mimics what happens with core decompression. Biologically, core decompression helps to reduce intra-osseous pressure and allows angiogenesis to revascularise the subchondral bone. However, the residual bone and marrow next to the bone tunnel after core decompression might also be osteopenic, owing to the administration of corticosteroids.

In order to test the hypothesis that corticosteroid therapy might adversely affect bone healing, we used an established model of steroid-associated osteonecrosis in rabbits to investigate bone healing in the mainly trabecular bone site, within a bone tunnel after core decompression at the distal femur.
Materials and Methods

We used ten 28-week-old male New Zealand white rabbits. The experimental protocol was approved by the Animal Experiment Ethics Committee of the Chinese University of Hong Kong. In five rabbits osteonecrosis was established by the administration of steroids according to our published protocol. Briefly, one injection of 10 μg/kg of lipopolysaccharide was given intravenously, followed after 24 hours by three intramuscular injections of 20 mg/kg methylprednisolone given at 24-hour intervals. We have shown previously that after two weeks, osteonecrosis will have occurred at several skeletal sites, including both the proximal and distal femur. Under general anaesthesia with intramuscular xylazine (2 mg/kg) and ketamine (50 mg/kg), core decompression was performed by drilling a 3 mm diameter hole bilaterally in the coronal plane of the distal femora two weeks after the last dose of steroids. The remaining five rabbits were injected with saline without receiving steroids and served as controls. Post-operative analgesia was provided with subcutaneous temgesic (0.02 mg/kg to 0.05 mg/kg) every six to 12 hours for up to three days. Micro-CT evaluations were performed in vivo at four, eight and 12 weeks post-operatively. The animals were killed after 12 weeks, at which point conventional histological and histomorphometrical analysis and biomechanical testing were performed for evaluation of bone healing within and around the bone tunnel after core decompression.

Micro-CT analysis of new bone formation in the bone tunnel.

The samples (n = 10 femurs in each group) were scanned using in vivo micro-CT, a high-resolution peripheral CT scanner (XtremeCT; Scanco Medical, Brüttisellen, Switzerland) with a spatial resolution of 40 μm according to the protocol for animal studies. The structure of the newly formed trabecular bone within the volume of interest of the entire bone tunnel was evaluated by the workstation with the built-in XtremeCT software. The mean volumetric bone mineral density (vBMD, mg/cm³), bone mineral content (BMC, mg/cm³), bone tissue volume density (BV/TV, %), connectivity density (Conn.D, 1 mm⁻³), trabecular number (Tb.N, 1 mm⁻¹), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm) and structure model index (SMI) to define the degree of the plate-like and rod-like trabecular bone, in the bone tunnel were measured. The XtremeCT finite element model was constructed directly from the segmented micro-CT data, applying a voxel-based a priori assumption of uniform bone mineralisation, which is recognised as an intrinsic limitation in obtaining accurate data to study woven bone with non-homogeneous mineralisation. A total of 160 slices of the bone tunnel were reconstructed into a three-dimensional (3D) image and an element-by-element finite element solver was used to perform uniaxial compression simulations for finite element analysis (FEA). Under compression strength of 10 GPa and a Poisson’s ratio of 0.3 from the distal surface and along the femoral axis, which mimicked the physiological load direction in the living rabbit, two variables in each sample were computed to calculate both bone stiffness (N/mm) and load to failure (N).

Descriptive histology. After micro-CT scanning, the distal femora were bisected along the midsagittal plane using a fine hacksaw. The medial halves were decalcified in 9% buffered formalin for two weeks, with the acid changed every two days. After gradient dehydration using 70% to 100% alcohol, the samples were embedded in paraffin. Longitudinal sections of the specimens 7 μm thick were prepared and stained with haematoxylin and eosin. A polarised light microscope (Leica Q500MC; Leica Microsystems, Wetzlar, Germany) was used to evaluate new bone matrix and the composition of the marrow within and around the bone tunnel, for both the surrounding residual bone and the subchondral area. The number of osteoclasts and the osteoblast perimeter percentage of the new bone formed in the bone tunnel were calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, Maryland). A total of ten regions in the middle of the tunnel were selected and ten slices in each region were evaluated under ×40 magnification to count osteoclasts and calculate their average number in the tunnel. The mean osteoclastic perimeter percentage was calculated by the ratio of the length of the trabecular surface covered by osteoclasts to the whole length of the trabecular surface in the bone tunnel. The measurements were performed with the examiners (ZL, DY) blinded to the group to which the sample belonged.

Mechanical test analysis. The lateral halves of the sagittally divided distal femora in each group were prepared to an identical thickness to permit a compression test using a 2.5 mm diameter indenter to obtain a surrogate index of the healing quality of new bone around the tunnel. After positioning the bone tunnel under radiological control to ensure that results were obtained without including the surrounding residual bone, an H25K-S material test machine (Hounsfield Test Equipment Ltd, Redhill, United Kingdom) was used to compress the bone at a rate of 10 mm/min to record Young’s modulus (MPa), stiffness (N/mm) and energy (10⁻³J).

Statistical analysis. All quantitative data were expressed as mean and standard error (SE). The distribution of data was tested for normality using the Shapiro-Wilk test and the p-value was found to be > 0.05, suggesting a normal distribution. Analysis of variance was used to compare changes of micro-CT parameters at different healing time points, and Student’s t-test was used to compare mechanical properties at the last healing time point at 12 weeks. SPSS version 10.0 (SPSS Inc., Chicago, Illinois) was used for statistical analysis and the level of significance was set at p < 0.05.

Results

All animals survived until they were killed at 12 weeks. There were no problems with wound healing around the operation site in any animal.
Micro-CT quantification of bone repair within the bone tunnel. Figure 1 shows the 3D structure of the new bone formed in the tunnel in both the osteonecrosis and control groups at week 12 after core decompression. The histomorphometrical data of the new bone formed within the tunnel of both groups at four, eight and 12 weeks are shown in Table I. Statistically significant differences in healing over time were noted, which implied that new bone formation increased towards the centre of the tunnel with time up to 12 weeks in both groups. Within each group, the BMD, BMC, BV/TV and Tb.Th were significantly higher at week 12 than those at week four (Table I, p < 0.05 for all).

At week four, new bone started to form, mainly next to the residual bone around the edge of the tunnel in both groups. Post hoc analysis showed that at week four, although the BMD, BV/TV, Conn.D and Tb.Th in the osteonecrosis group were lower than those in the control group, such differences were not statistically significant, except for Tb.Th (BMD, p = 0.06; BV/TV, p = 0.14; Conn.D, p = 0.224; Tb.Th, p = 0.045). At week eight, the BMC, BV/TV, Conn.D and Tb.Th increased in both groups compared with the results at week four, and the BV/TV, Conn.D and Tb.Th in the osteonecrosis group were significantly lower than those in the control group (BV/TV, p = 0.004; Conn.D, p = 0.005; Tb.Th, p = 0.035). At week 12, there was no difference for BMD, BMC and TB.Sp between the two groups, but the BV/TV, Conn.D, Tb.N and Tb.Th in the osteonecrosis group were significantly lower than those in the control group (BV/TV, p = 0.012; Conn.D, p = 0.016; Tb.N, p = 0.048; Tb.Th, p = 0.029). The SMI in the osteonecrosis group was significantly lower than that in control group (p = 0.037).

Simulated compression test results of micro-CT finite element analysis. The simulated compression along the femoral long axis in a distal to proximal direction showed that the compression stiffness and the load to failure of the distal femora in the osteonecrosis group were significantly lower than those in the control group stiffness, p = 0.048; load to failure, p = 0.00; Table II).

Descriptive histomorphology of new bone formed in the bone tunnel. The histomorphological appearance of bone healing in the tunnel at week 12 is shown in Figure 2. The centre of the tunnel in the control group was filled with normal bone marrow, and newly formed bone had mostly accumulated at an area about 1 mm from the margin of the tunnel. The new bone was thicker and well connected to the residual bone adjacent to the tunnel. It showed a normal woven structure with osteocytes embedded in the trabecular matrix and some osteoblasts lining the new bone surface, with no empty lacunae or apoptosis of osteocytes. In the osteonecrosis group, the tunnel was filled with abundant fibrous tissue, bone marrow infiltrated with mononuclear cells, osteoclasts and some scattered fragments of new bone. Although no empty lacunae were found, the new bone was thin with more active osteoblasts and osteoclasts. The bone histomorphometry showed that the mean number of osteoclasts (Oc.N) and the osteoblast perimeter percentage (Ob.Pm) in the bone tunnel of the osteonecrosis group were significantly higher than those in the control group (Table III, Oc.N, p = 0.000; Ob.Pm, p = 0.039). Polarising microscopy showed coherent alignment of collagen fibres along the direction of the newly formed bone in the control group, but this remained disorganised in the osteonecrosis group.

Descriptive histomorphology of the residual bone around the bone tunnel. We examined the bone surrounding the tunnel approximately 1 mm from the edge (Fig. 3) at week 12. In the osteonecrosis group, the bone marrow around the tunnel was infiltrated by a number of mononuclear cells and few osteoclasts, with empty lacunae in trabeculae surrounded by ongoing reparative osteogenesis. The collagen fibres of the surrounding trabecular bone demonstrated a regular lamellar pattern under polarising microscopy. Normal bone and marrow were seen in the control group, characterised by regular osteocytes in the bone matrix, osteoblasts lining the bony surface, and regular alignment of the collagen fibres in a lamellar pattern.

Descriptive histomorphology of the subchondral bone. No fracture or collapse was found in the subchondral bone near the tunnel at week 12 (Fig. 4). In the osteonecrosis group, a number of empty lacunae and scattered osteocytes were observed in bone trabeculae, with few osteoblasts lining the surface, whereas in the control group normal distribution and morphology of osteocytes and osteoblasts were found.
Mechanical test. The compression test showed that at week 12, Young’s modulus and stiffness in the osteonecrosis group were significantly lower than that of the control group (Table IV; Young’s modulus, \( p = 0.018 \); stiffness, \( p = 0.011 \)).

Discussion
This study used in vivo micro-CT, conventional histology and histomorphometry, radiography and mechanical testing to systematically investigate bone repair after core decompression in rabbits with steroid-induced osteonecrosis, in order to understand the characteristics of impaired bone healing following steroid administration.

Compared with a control group, we demonstrated that the micro-CT parameters BV/TV, Conn.D, Tb.N and Tb.Th of the new bone formed in the tunnel were significantly lower in the osteonecrosis group, whereas SMI was higher. This is a structural index for trabecular bone. An SMI = 0 signifies the plate-like and intact trabecular bone, whereas SMI = 3 means the rod-like or osteoporotic trabecular bone in intact bone. In this study, the SMI results showed that the amount of rod-like or immature trabecular...
Histological images of the new bone in the bone tunnels at 12 weeks after core decompression in a) the osteonecrosis group, showing fibrous connective tissue (black arrows) alongside newly formed bone and marrow, with scattered osteoclasts (blue arrows) in the bone marrow. Numerous mononuclear cells had infiltrated into the bone marrow, apart from fibrous connective tissue and this trabeculae with more active osteoblasts lying on its surface (haematoxylin and eosin, × 20), and in b) the control group, showing normal newly formed bone marrow and trabecular bone, with numerous osteoblasts along the trabecular surface (black arrows) and regular bone marrow fat cells (haematoxylin and eosin, × 10).

Table III. Number of osteoclasts per section (3 mm in diameter) and osteoblast perimeter percentage in the bone tunnel at week 12 after core decompression: mean values (standard error)

<table>
<thead>
<tr>
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<th>Osteonecrosis group</th>
<th>Control group</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Oc.N*</td>
<td>18.83 (3.76)</td>
<td>4.66 (1.21)</td>
<td>0.000</td>
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<tr>
<td>Ob.Pm† (%)</td>
<td>80.42 (10.35)</td>
<td>66.93 (9.82)</td>
<td>0.039</td>
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* Oc.N, number of osteoclasts
† Ob.Pm, osteoblast perimeter percentage

Bone in the osteonecrosis group, when used to study healing, was higher than that in the control group. Although no significant difference existed in the mean BMD between the groups (p = 0.076), we found that the mean BMD in the control group was a little higher at each interval in the study. Impaired or delayed osteogenesis and remodelling of new bone in the tunnel was demonstrated histologically in the osteonecrosis group. The bone defects were created in the distal femora of rabbits because osteonecrosis had been reported in that region. This position was convenient for surgical intervention and follow-up evaluations. The empty lacunae and the reparative osteogenesis in the surrounding area of the tunnel in the distal femora confirmed that steroid-induced osteonecrosis had occurred, which would impair bone healing in the early stages after administration of steroids. It was reported that the glucocorticoid-induced impairment of osteoblast, osteocyte and osteoclast function led to reduced bone remodelling and diminished potential for repair of damaged bone. The osteogenic ability of marrow-derived mesenchymal stem cells also decreased during the early steroid-induced osteonecrosis development found in our previous work, and also reported by others. The smaller volume and reduced thickness of new trabecular bone in the bone tunnel in the osteonecrosis group in the early stage might be due to reduced osteogenic ability and bone remodelling in the residual bone around the tunnel.

Healing in normal or osteoporotic bone has been extensively examined, both clinically and experimentally. Little work has evaluated the potential adverse effects of corticosteroids on fracture healing in the epiphyseal and metaphyseal regions, where trabecular bone is mainly present, a location recently reported to have a high incidence of steroid-associated fractures in patients with systemic lupus erythematosus. Systemic steroid administration has been reported to inhibit bone healing in a rabbit model of ulnar osteotomy. However, other work has not revealed inhibitory effects on healing in the long bones of rats after the short-term administration of prednisolone. This difference might be due to variations in the dose and duration of steroid administration. In our study, short-term and pulsed steroid administration was performed at an early stage, but the healing in the tunnel after core decompression was evaluated 12 weeks after steroid induction. The results revealed that the volume, thickness and density of new bone formed in the tunnel in the
Histological images of the bone tissue and marrow around the bone tunnels at 12 weeks after core decompression in a) the osteonecrosis group, showing numerous mononuclear cells (yellow arrows) infiltrating the bone marrow and also several empty lacunae (black arrows) in the bone trabeculae surrounded by reparative osteogenesis (blue arrows), and in b) the control group, showing mature bony tissue and bone marrow with mature and thick trabeculae and numerous osteocytes (black arrows) in the bone matrix, but fewer osteoblasts lining the trabecular surface (haematoxylin & eosin, × 20).

Histological images of bone tissue at 12 weeks, in a) the osteonecrosis group, showing necrotic bone tissue (ON) beneath the articular cartilage with empty lacunae (black arrows) and osteoblasts (yellow arrows) surrounding the surface of the subchondral bone but less around reparative osteogenesis (black dotted arrows), and b) the control group, showing normal subchondral bone with numerous lacunae and normal osteocytes (black arrows) in the matrix and osteoblasts (yellow arrows) lining the surface of the subchondral bone (haematoxylin and eosin, × 20).

| Table IV. Mechanical analysis of newly formed bone in the bone tunnel at week 12 after core decompression: mean values (standard error) |
|---------------------------------|-------------------|------------------|-----------------|
|                                 | Osteonecrosis group | Control group    | p-value         |
| Young's modulus (MPa)           | 6.20 (2.54)        | 14.77 (1.05)     | 0.018           |
| Stiffness (N/mm)                | 22.18 (2.96)       | 68.42 (13.38)    | 0.011           |
| Energy (10^3J)                  | 21.53 (6.03)       | 15.60 (6.69)     |                 |

The osteonecrosis group were lower than those in the control group at four, eight, and 12 weeks, which would be explained by the reduced osteogenic capability of existing mesenchymal stem cells. Thus steroids could also inhibit healing in a defect in predominantly trabecular bone, suggesting a strong need for intervention or augmenting bone healing in patients receiving corticosteroids, especially those with fractures in mainly trabecular bone, such as the spine and hip or patients with osteonecrosis who have undergone core decompression.
This study also showed that the osteonecrosis group had significantly inferior mechanical properties in terms of compression load and Young's modulus compared to the control group. This finding was also supported by FEA from a micro-CT simulated compression test at the distal femur. The mechanical test showed that the stiffness of new bone in the bone tunnel in the osteonecrosis group was significantly lower than that in the control group. Although the compression test used in this study examined mainly the mechanical properties of the healing interface between new bone in the tunnel and the residual bone around it, it suggests indirectly that the new bone formed in the tunnel in the osteonecrosis group has poor mechanical properties. This could also imply that the risk of joint collapse in patients with osteonecrosis of the hip is increased when a relatively large piece of necrotic bone is removed at core decompression. The simulated compression test using FEA also showed that the stiffness and the load to failure of the distal femur of rabbits in the osteonecrosis group were lower than those in the control group. Our results seemed similar to those from clinical studies in which patients with osteonecrosis had a significantly higher rate of mechanical failure than those without.40 The low volume of bone formed in the tunnel and the relatively low BMC might explain these inferior mechanical properties.

This is the first experimental study to systematically demonstrate impaired or delayed bone healing in a model of trabecular bone defect in rabbits with steroid-induced osteonecrosis. This model might be used for developing treatment strategies to improve the prognosis in bone healing in defects resulting from the administration of steroids.

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References

