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Metabolic Disorders and Mineral Density of the Bone Tissue in the Early Pathogenesis of Osteonecrosis: Study on Rabbits with Steroid-Induced Osteonecrosis

L. Wang[1], L. Zhang[2], and M. Gao[3]

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The relationship between the appearance of bone metabolism disorders and the onset of steroid-induced osteonecrosis remains unclear. We studied the time course of calcium, phosphorus, osteocalcin, alkaline phosphatase, and mineral density of bone tissue in the subchondral bone of the femoral head of rabbits injected with steroids and attempted to precisely determine the time when disorders in bone metabolism started in animals with steroid-induced osteonecrosis. We detected bone metabolism disorders involved in the early pathogenesis of steroid-induced osteonecrosis, which were the cause, but not the result of this condition.

Key Words: *steroid drug; osteonecrosis; mineral; bone tissue mineral density*

Inorganic components of the bone, mainly calcium and phosphorus, are essential for bone formation and development at the molecular level. Imbalance between bone resorption and bone formation leads to a disease, such as nontraumatic osteonecrosis (NON). One of the most frequent factors fraught with NON is steroid therapy for some diseases, for example, severe acute respiratory syndrome (SARS) [9], systemic lupus erythematosus, *etc.* Steroid excess can directly affect bone cells: it reduces osteoblast and osteoclast production, suppresses production of osteoblast and osteoclast precursor, stimulates apoptosis of osteoblasts and osteocytes, and prolongs osteoclast life span [6]. The balance between bone tissue resorption and formation is disturbed in NON, which leads to the development of abnormal structure of the bone and its dysfunction resulting in femoral head shrinkage eventuating in destruction of the femoral bone [5,6,8]. The period between the appearance of bone metabolism disorders and onset of NON is still not determined. The significance of the initial disorders in the bone tissue metabolism and the relationship between initiation of these disorders and steroid-induced NON development remain not quite clear.

We studied changes in the levels of serum calcium, phosphorus, osteocalcin, alkaline phosphatase (AP) and bone mineral density (BMD) of the subchondral bone of the femoral head and evaluate the time of development of bone tissue metabolism disorders in steroid-induced NON in rabbits injected with steroids [5].

MATERIALS AND METHODS

Animal model. All rabbits were kept under standard conditions at optimal temperature in pathogen-free rooms with free access to water and standard fodder. The protocol of experiment was approved by the Ethic Committee of Shenzhen Institute of Advanced Technology (No. SIAT-IRB-140110-YYS-WLEI-A0010). Experiments were carried out at Center for Animal Research, Shenzhen Institute of Advanced Technology, with due consideration for the philosophy of humane attitude to animals.

The study was carried out on adult female Japanese albino rabbits (n=55) weighing 3.5 kg (mean age

[1]Shenzhen Institute of Advanced Technology, Chinese Academy of Science, Shenzhen, China; [2]Faculty of Health Sciences, University of Macau, Macau, China; [3]Department of Orthopedic Surgery, The 2nd People's Hospital of Shenzhen, Shenzhen, China. ***Address for correspondence:*** wldzzs@163.com. L. Wang

24 months). The animals were distributed at random into three groups for three periods (3, 7, and 14 days). All animals received a single dose of methylprednisolone acetate (MP; Pfizer Manufacturing Belgium NV) intramuscularly (4 mg/kg). Controls (n=10) were kept under identical conditions and received no steroids.

Serum levels of calcium, phosphorus, osteocalcin, and AP. Venous blood specimens were collected in all rabbits, serum was separated and stored at -80°C until analysis. Serum calcium was measured by the methylthymol blue (MTB) test, phosphorus with the use of molybdic acid, AP with the use of disodium phenylphosphate, and osteocalcin concentration was evaluated using commercial ELISA kit (all kits were from Jiancheng Biotechnology Company).

Evaluation of subchondral bone BMD. Directly after the animals were sacrificed under deep anesthesia, the femoral head was isolated from two sides and fixed in 10% neutral buffered formalin. Shifts in BMD of the subchondral bone of resected femoral head were detected using a SkyScan 1076 micro-CT scanner (Bruker) and CTAn software (SkyScan), with the isotropic voxel=17.33 μm.

Histopathological studies. After micro-CT scanning, the femoral head was decalcified with EDTA, embedded in paraffin, and sliced into 4-μm sections

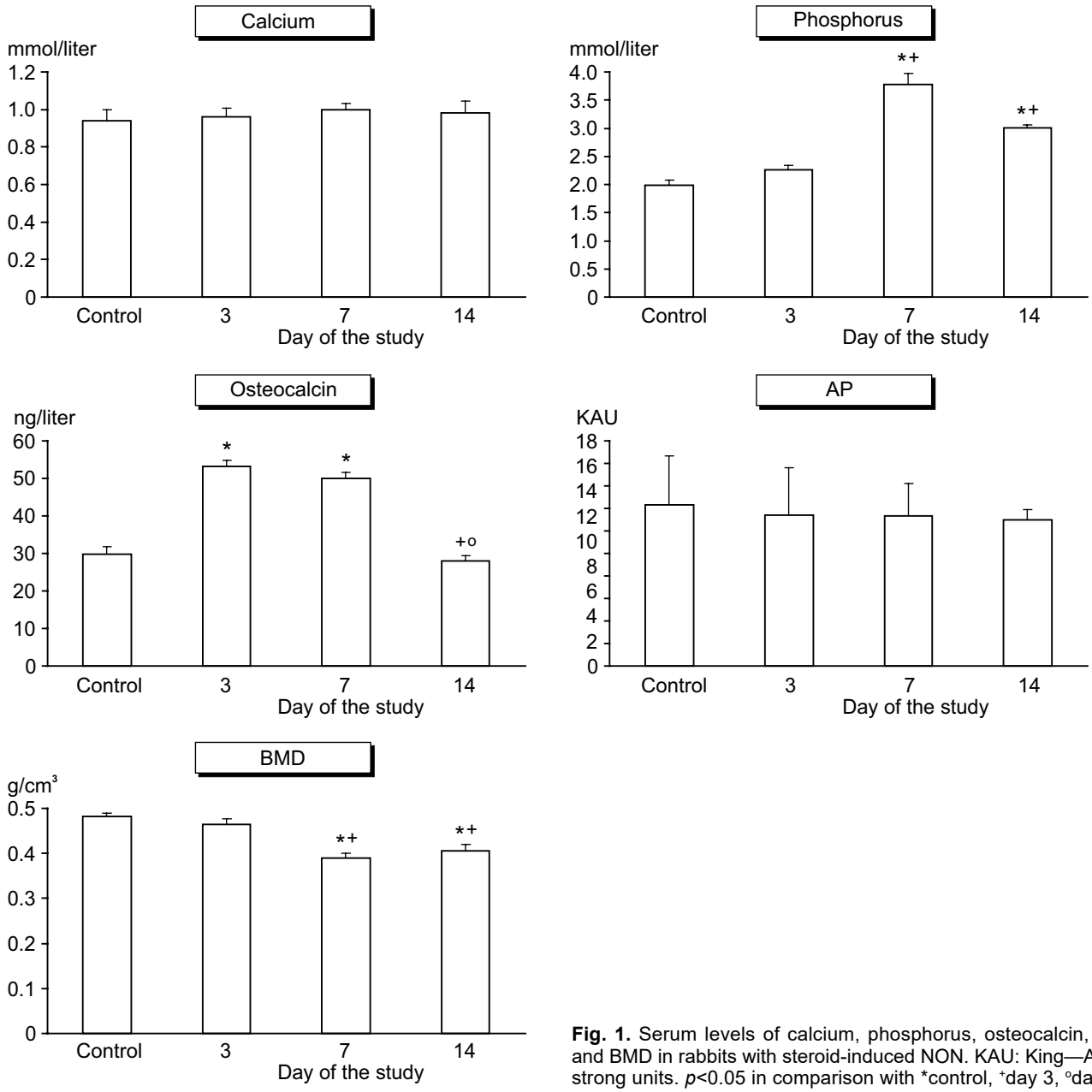

Fig. 1. Serum levels of calcium, phosphorus, osteocalcin, AP, and BMD in rabbits with steroid-induced NON. KAU: King—Armstrong units. p<0.05 in comparison with *control, $^{+}$day 3, ^{o}day 7.

in the frontal plane; the sections were stained with hematoxylin and eosin. The bone and bone marrow tissues were examined under a light microscope. The NON involvement was evaluated using previously defined criteria [5]. NON was diagnosed in cases with diffusely scattered empty lacunas or presence of osteocyte pyknotic nuclei in bone trabeculae associated with necrosis of bone marrow cells. The incidence of NON for each period of the study was estimated as the proportion of the number of rabbits with NON to total number of animals.

Statistical analysis. The data were processed using SPSS 16.0 software (IBM) with ANOVA and *t* tests for related variables, Student—Newman—Keuls (SNK) multiple range tests, and the least significant difference (LSD) test for intergroup comparisons. The difference in NON incidence in the groups was evaluated using Fisher's precise test. All data were presented as $M \pm SD$. The differences were significant at $p<0.05$.

RESULTS

Serum calcium and phosphorus levels. Serum calcium level increased in 3, 7, and 14 days after injection of steroid in comparison with the control (Fig. 1), plasma phosphorus level increased significantly after 7 days and decreased after 14 days. The femoral head subchondral bone BMD decreased on days 7-14 in comparison with the control group. It had been proven [1] that steroid therapy caused bone demineralization. Our study confirmed that steroid-induced fluctuations in calcium and phosphorus levels could promote bone demineralization.

Serum osteocalcin concentration. The concentration of osteocalcin was higher on days 3 and 7, after which it decreased (day 14 after steroid injection; Fig. 1). Our results were not consistent with a previous report [4] demonstrating a decrease of serum osteocalcin concentration to the levels beyond the sensitivity of analysis within 3 days of steroid treatment, after which it increased by week 3, however, remaining at the level of 50% of its initial level [4]. However, these authors did not explain why serum osteocalcin level increased 3 days after steroid treatment. Osteocalcin was produced and secreted by mature osteoblasts, the greater part of produced osteocalcin is deposited in the bone matrix, while the remaining 20% are released into the blood [3]. Serum osteocalcin level positively correlated with osteoblast synthesis. We hypothesized that the increase of osteocalcin level was the response of mature osteoblasts to steroids under conditions of initial disorders of bone metabolism after steroid-induced NON, after which the level of mature osteoblasts decreased, causing a decrease of osteocalcin level. Steroid excess can directly influence bone cells by reducing the production of osteoblasts, suppressing the production of osteoblast precursors, and stimulating osteoblast apoptosis [6].

Serum AP level. AP is a glycoprotein enzyme related to osteoblast membrane. AP is released into circulation during bone formation phase in the course of remodeling. It is also an early and important indicator of bone formation and bone tissue regeneration [2]. Our data (Fig. 1) indicated a decrease of serum AP concentration on days 3-14, which was in line with the results of a previous report [7] demonstrating a significant reduction of AP activity in the rat femoral diaphysis in response to hydrocortisone injection. This decrease could be due to inhibitory effects of steroids on the metabolic functions in osteoblasts.

On the whole, serum levels of phosphorus, osteocalcin, and BMD demonstrated significant shifts 7 days after steroid injection in comparison with the control group. In just solitary cases serum osteocalcin level changed significantly within the first 3 days in comparison with control.

Histopathological study. NON was not observed within 3 days after steroid injection and was detectable only after 7 days, manifesting only after 14 days

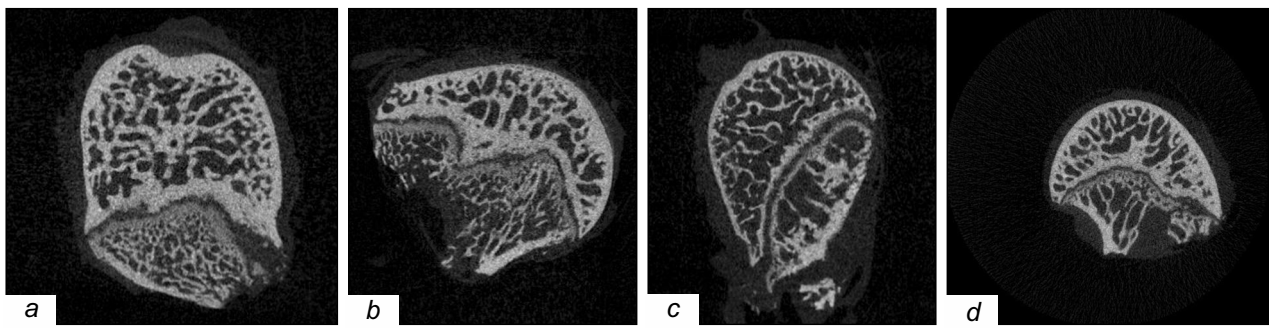

Fig. 2. 2D images of resected femoral head: control (*a*) and experimental rabbits on day 3 (*b*), 7 (*c*), and 14 (*d*). *a*, *b*) No bone or marrow necrosis; *c*, *d*) necrotic areas presented by accumulations of degenerative or necrotic medullar hemopoietic cells; bone trabeculae with necrotic changes: empty lacunas and condensed nuclei in osteocytes.

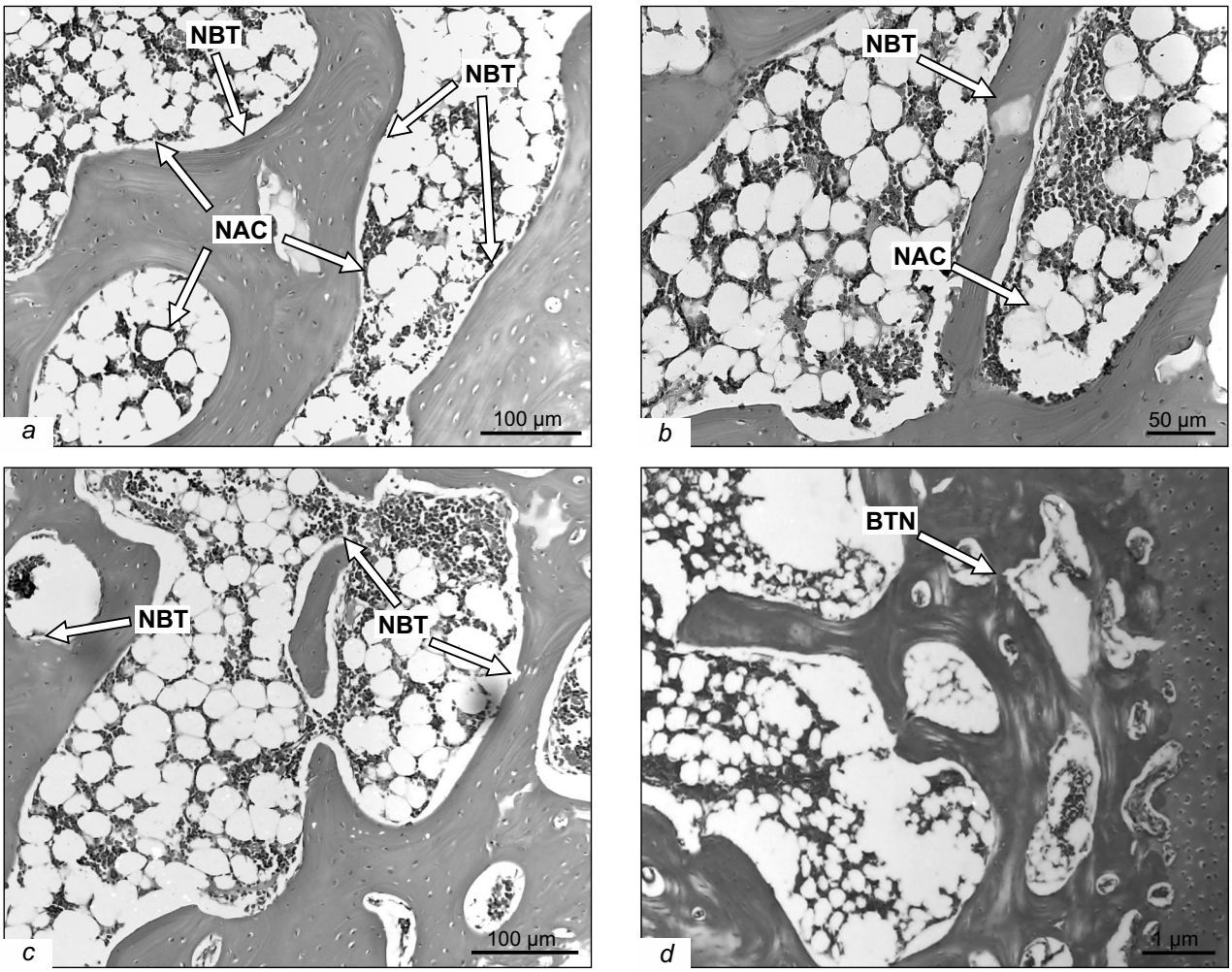

Fig. 3. Histopathological sections of femoral head: control (*a*) and experimental groups on days 3 (*b*), 7 (*c*), and 14 (*d*). Hematoxylin and eosin staining. *a*, *b*) No bone or bone marrow necrosis. NAC: normal adipocytes; NBT: normal bone trabeculae. *c*, *d*) Necrotic foci presented by accumulations of degenerative or necrotic medullar hemopoietic cells; bone trabeculae also characterized by necrotic changes: empty lacunas and condensed nuclei in osteocytes. BTN: bone trabeculae with necrosis.

(Figs. 2, 3). The incidence of NON was insignificant (p=0.2635) on day 7 (27%) and on day 14 (153%), which was in line with our previous data and results of other authors [5,8].

Thus, serum osteocalcin levels, changing significantly 3 days after steroid treatment in comparison with the control group, despite the 27% incidence of NON on day 7, suggest that changes in the bone metabolism unfolding before NON are involved in the early pathogenesis of steroid-induced form of this disease and serve as its cause, but not the result. Our results promoted better understanding of the processes underlying NON development. This study was carried out on just a small sample, and we did not evaluate bone resorption markers. In addition, the molecular and cellular mechanisms of NON initiation remain little studied, but we hope, further studies will help us find answers to these questions.

L. Wang and L. Zhang made an equal contribution to the study.

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