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An animal model of femoral head osteonecrosis induced by a single injection of absolute alcohol: An experimental study

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Background:

The lack of an experimental animal model that can reliably mimic all stages of osteonecrosis of the femoral head has hindered progress toward the successful prevention and treatment of the disease.

Material/Methods:

A goat model of osteonecrosis of the femoral head (ONFH) was established and observed from the early to the intermediate-to-late stage of mechanical failure. Absolute alcohol was injected slowly into the center of bilateral femoral heads in 12 adult Small Tail Han goats. Postoperatively, the femoral heads were harvested and examined using macrostructural and histological analyses and radiographic and MRI examinations at weeks 4, 8, 12, and 25.

Results:

Macrostructural and radiographic examinations revealed that the contour of both femoral heads was deformed slightly at 12 weeks, but a contour deformation with joint space narrowing was observed at 25 weeks. Histologically, a strong concordance with the natural history of ONFH in humans was found. The present model demonstrated bone trabeculae, marrow necrosis, a reconstruction deficiency and destruction of the microcirculation.

Conclusions:

Among quadrupedal models, the goat model of ONFH, which is induced by a single injection of absolute alcohol, may be suitable and valuable for the evaluation of various therapeutics and side effects in the treatment of ONFH.

Key words:

osteonecrosis of the femoral head • alcohol • animal model • goat • mechanical failure

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BACKGROUND

Osteonecrosis of the femoral head (ONFH) is diagnosed with increasing frequency in young adults and has a significant socioeconomic impact [1]. Despite recent diagnostic advances, effective treatments have been elusive, and the majority of ONFH cases ultimately result in a collapse of the femoral head. Most surgical therapies aim to prevent collapse of the subchondral bone, but the reported clinical results are inconsistent. Surgical methods and curative effects are difficult to define because the pathogenesis of ONFH is unknown [2,3]. The lack of an experimental animal model that can reliably mimic all stages of the human disease [4] has hindered progress toward the successful prevention and treatment of ONFH.

In the present study, absolute alcohol was injected into the center of bilateral femoral heads, which could histopathologically mimic the disorder from early to intermediate-to-late stages of mechanical failure. The purpose of this study was to provide an animal model to test potential surgical interventions and new therapeutic approaches.

MATERIAL AND METHODS

The local Animal Care and Use Committee approved the following experimental protocol. Sixteen adult female Small Tail Han goats (age, 24–36 months; weight, 50–65 kg) were used. Twelve goats were fasted for 12 h before surgery. The goats were premedicated with ketamine (10 mg/kg) intramuscularly 10 min before induction of anesthesia with pentobarbital sodium (30 mg/kg) intravenously. The goats were placed in the right lateral position. The left hip region was shorn, and the skin was cleaned. Next, the skin was disinfected, and the left limb was prepared using standard aseptic techniques. An 8 cm posterolateral arc incision was made in the region below the left greater trochanter while carefully protecting the surrounding soft tissue. Under X-ray guidance, a 5 to 5.5 cm tunnel was drilled with a 3 mm diameter Steinman pin into the center of the femoral head. The Steinman pin was withdrawn and replaced with a hollow tube. Ten milliliters of absolute alcohol (>99.7%, v/v) was injected slowly (0.5 ml/min) through the tube, and after 30 min, the hollow tube was removed. The foramen was sealed with bone wax (Ethicon). Finally, the surgical field was irrigated with warm saline solution, and the incision was closed in layers. The right hip was injected using a technique similar to that described for the left hip. Postoperatively, all animals were injected intramuscularly with penicillin as prophylaxis. During the postoperative period, the goats were allowed to roam freely in an indoor-outdoor enclosure. The other 4 Small Tail Han goats were used as normal controls (1 per time point). Three experimental goats and 1 control goat were killed at postoperative weeks 4, 8, 12, and 25.

Macroscopic examination

The surface of the cartilage and the bone-tunnel were observed after preparation of the samples following euthanization.

X-ray examination

The general contours of the femoral head and hip articulation were observed by anteroposterior pelvic imaging.

MRI examination

Bilateral acetabula and femoral head images of the experimental goats were obtained at week 25 using MR scanning with 5 mm cross and coronal sections (including T₁W, T₂W and STIR).

Histological analysis

The femoral heads were sectioned into 5 mm thick slices parallel to the femoral shaft. The tissue was fixed in 10% formalin solution for 1 week. The slices were then decalcified in 10% EDTA solution for 2 weeks and cut into 4 uniform parts. The samples were embedded in paraffin, and 5 μm thick sections were generated using a microtome. The sections were stained with hematoxylin-eosin (HE) and Masson trichrome and examined under a light microscope. The histological images were converted to grayscale images using a computer to calculate the percentage of empty lacunae and the expression rate of collagen fibers.

Statistical analysis

All numerical data are presented as the mean ± standard deviation (SD). The difference between the 2 groups was calculated with the Student *t*-test. Differences of multiple groups were calculated with one-way ANOVA (SAS Ver. 8.1). Values were considered significant when *p*<0.05.

RESULTS

Macroscopic examination

At 4 and 8 weeks postoperatively, the articular surfaces and the femoral heads were intact. Osteonecrosis was observed around the drill track and demonstrated a decrease with an increase in distance to the tip of the drill track. At 12 weeks, 3 experimental goats were examined, and 5 articular surfaces demonstrated a local loss of luster. The defect of the articular cartilage was observed at 25 weeks (Figure 1).

X-ray examination

At week 4, 12 experimental goats were examined, and mild cystic degeneration was detected in 20 femoral heads. At 8 weeks, the bone mineral density of the 17 femoral heads had increased locally in the 9 experimental goats. At 12 weeks, 6 experimental goats were examined, and an inhomogeneous trabecular bone density was observed in all 12 femoral heads; however, slight contour changes were found in 8 heads. At 25 weeks, the last 3 experimental goats were examined, and a local collapse and narrowing of the hip joint space was observed in 4 femoral heads (Figure 2A, B).

MRI examination

The last 3 experimental goats were examined at week 25. In the 4 femoral heads, high signals were obtained for a narrow hip joint space, local contour collapse, and intra-articular effusion using T₂-weighted and STIR imaging, and low signals were acquired using T₁-weighted imaging (Figure 3).

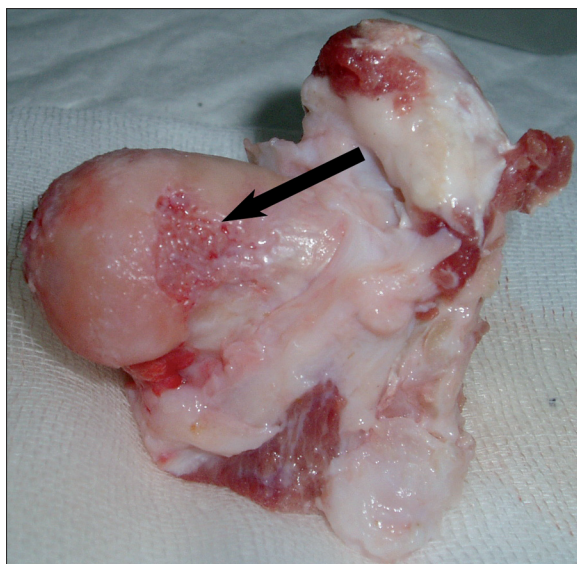


Figure 1. The contour of the femoral head was deformed at week 25. The joint surface was not smooth and cartilage was damaged.

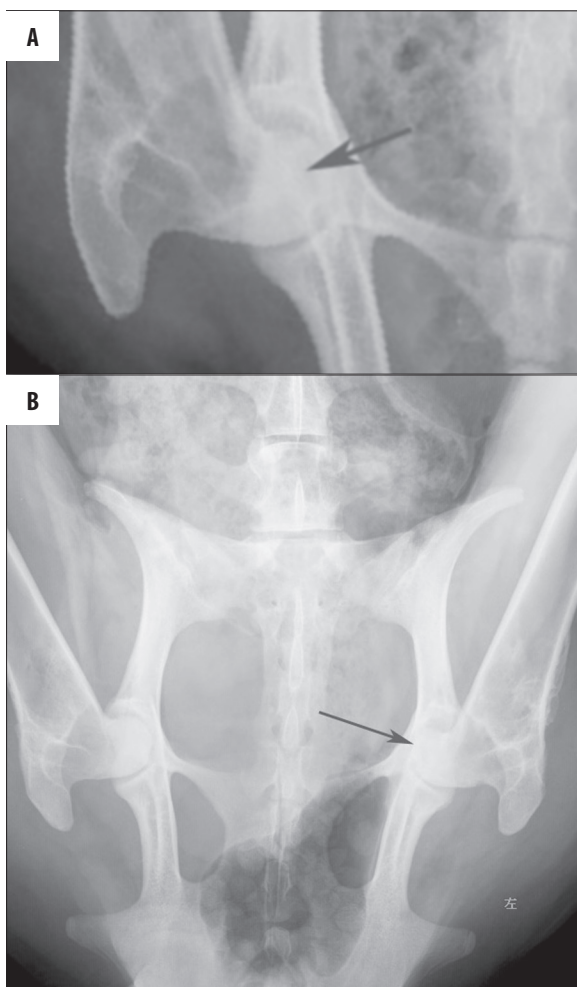


Figure 2. (A) X-ray examination showed slight cystic degeneration in the femoral head at week 4. However, the joint space was normal for both hips. (B) X-ray examination showed the femoral heads were not so round and narrowing of hip joint at week 25, which was more obvious at left as indicated.

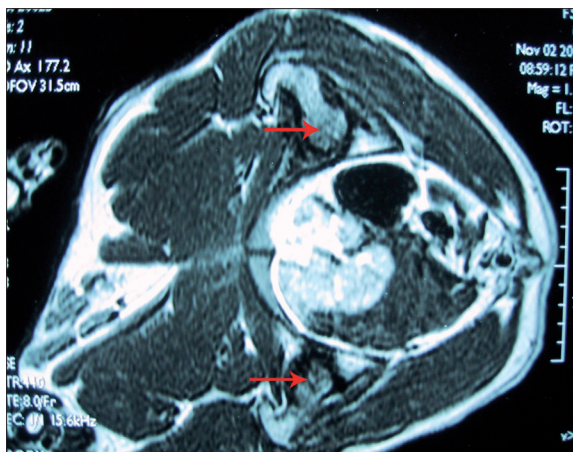


Figure 3. MRI showing bilateral femoral head osteonecrosis. The signal in the femoral head was characteristics of ongoing of necrosis and repairing.

Histological analysis

Four weeks

Many of the observed osteocytes were necrotic (>45% empty lacunae). Hematopoietic cells were absent, and a large number of erythrocytes had died in the marrow cavities. The nuclei of the swollen adipocytes had been lost. Sparse fibrous tissue and a modest amount of bone debris were observed in the marrow cavities, together with irregular trabecular resorption.

Eight weeks

In highly damaged necrotic areas (>60% empty lacunae), most of the marrow cavities were filled with fibrous tissue, which was characterized by a high level of cellularity with numerous macrophages. The necrotic trabecular bones had fractured. However, some new bone formation was detected and was surrounded peripherally by a large number of osteoprogenitor cells.

Twelve weeks

The percentage of empty lacunae had decreased slightly (>50%). In necrotic areas, the fibrous tissues had increased and thickened, and osteoclast proliferation and activation were observed. A number of new bones had formed, and the trabecular bones had become broader. However, the chondrocytes were dispersed and severely decreased in number.

Twenty-five weeks

The percentage of empty lacunae had decreased significantly (>35%). Massive amounts of trabecular bone had been absorbed. A large amount of bone debris and osteoclasts were detected in most of the marrow cavities, which were filled with dense fibrous tissues that exhibited a high level of cellularity with numerous fibrocytes. In the necrotic trabecular areas, active osteoblasts, bone debris and newly woven bone were present. Tenuous trabecular bone and microfractures were observed in the subchondral

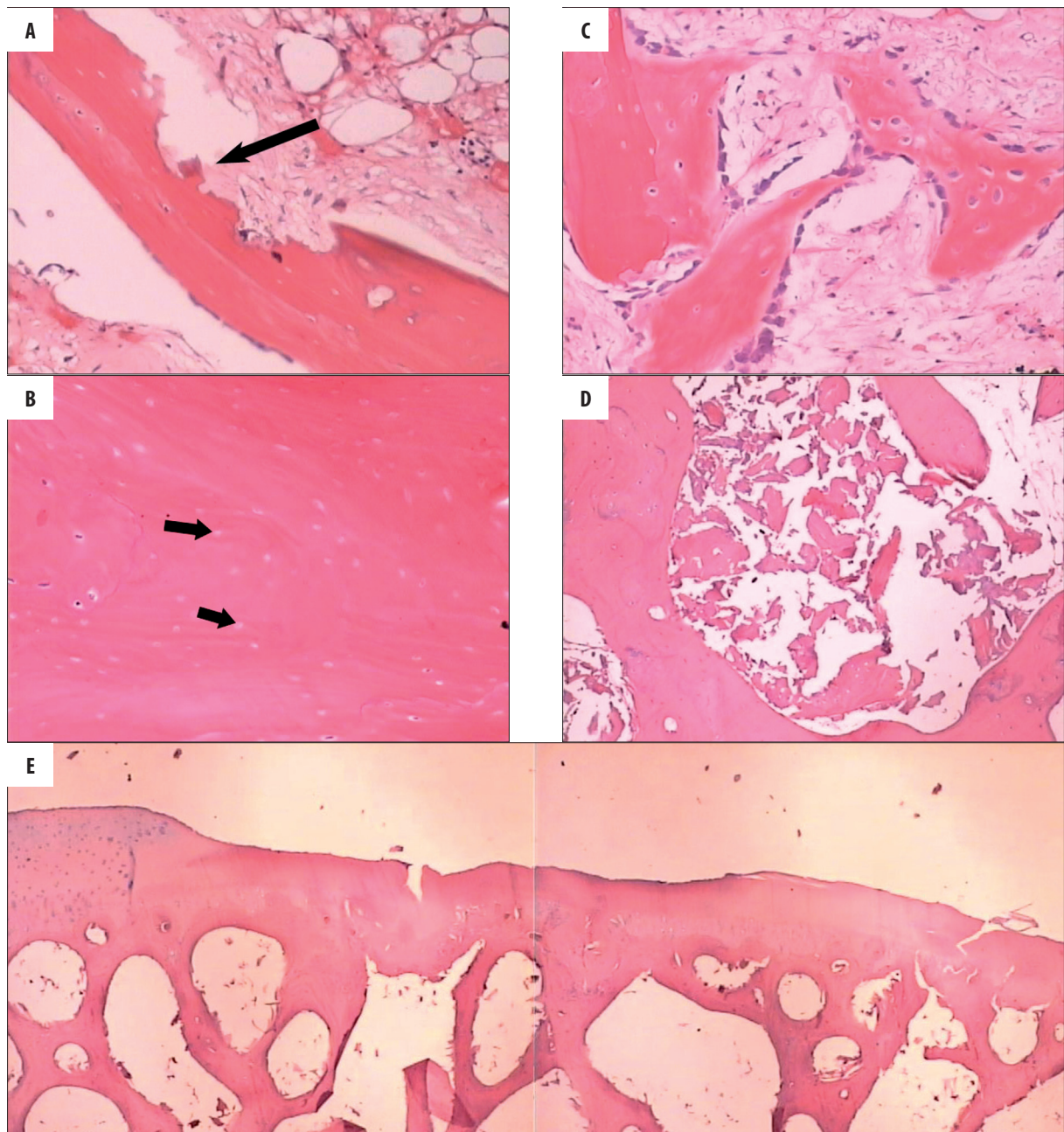


Figure 4. (A) Histological specimen showed damaged trabecular resorption at week 4 (HE, 40 \times). (B) A number of the osteocyte-filled lacunae were replaced by empty lacunae at week 8 (HE, 100 \times). (C) Fibrous tissue with some new bone formed at week 12 (HE, 100 \times). (D) Massive bone fragmentation was showed in the marrow cavities at week 25 (HE, 100 \times). (E) Deformation of joint contour and irregularity of the subchondral bone and loss of chondrocytes was showed at week 25 (HE, 20 \times).

bone. The number of chondrocytes had decreased severely, and these cells were almost absent in the collapsed area (Figure 4A–E).

Percentage of empty lacunae and expression of fibrosis

Twelve sections each of the femoral heads were obtained from the experimental and control groups. In these sections, 500 lacunae were measured randomly under low magnification (100 \times), and the number of empty lacunae was determined. The total number of empty lacunae in the control groups was used to increase the sample size, because

the time difference between the 4 control groups was not statistically significant ($p=0.3281$) (Table 1).

The sections were prepared as described previously to analyze the fibrous tissue quantitatively using an image analyzing system (Axioplan 2 imaging microscope). The images were input into the computer (1200 \times , 1300 \times 1030 pixels). Five random fields of view were assessed, and the mean value was determined. The numeric values for the 4 control groups were summed to increase the sample size since the time difference between the 4 control groups was not statistically significant ($p=0.8485$) (Table 2).

Table 1. Percentage of empty lacunae in the experimental groups and the control group ($\bar{x}\pm s$, CI).

| Group | Percentage of empty lacunae | Confidence Interval |
|---------------|-----------------------------|---------------------|
| Control group | 2.53±0.795 | 2.30~2.76 |
| 4 w | 47.23±3.492*,** | 45.02~49.45 |
| 8 w | 68.45±6.060*,** | 64.60~72.30 |
| 12 w | 54.94±5.648*,** | 51.35~58.53 |
| 25 w | 36.37±7.622*,** | 31.52~41.21 |

* Difference between the experimental group and the total control group was statistically significant ($p<0.0001$); ** difference between each experimental group was statistically significant ($p<0.05$).

DISCUSSION

Osteonecrosis of the femoral head was classified clinically into 2 major types of the condition according to the etiology – traumatic and non-traumatic. The factors that determine the pathological mechanism of femoral head osteonecrosis include pathogenesis, biomechanics and the balanced relationship between bone resorption and reconstruction. Previous studies utilizing animal models have focused on quadrupeds, including dogs [5–7], rabbits [8,9], rats [10,11], sheep [12], swine [13–15], horses [16], and goats [17]. Investigations of bipeds have included chickens [18] and emus [19,20]. The techniques used for induction are extensive and have included steroids injection [14,18,21], hyperbaric exposure [12], endotoxins injection [22,23], deep freeze insults [24], vessel ligation [11], hypersensitivity reactions [17], hip luxation [25], and various combinations of these insults [24,25]. Nonetheless, a classic animal model of femoral head osteonecrosis is not available. The histological changes comprising the conspicuous features of osteonecrosis can be mimicked with relative ease. However, until recently, most quadrupeds have generally failed to progress to late stage femoral head collapse, and aggressive systemic insults have led to cachexia or death [26]. Goats were selected for the present study because their hip and vascular anatomy is similar to humans. Absolute alcohol induces cellular dehydration, protein coagulation, vasoconstriction, endothelial cell degeneration and thrombosis. Ethanol injections have been employed therapeutically (e.g., injected into bone metastases, autologous bone neoplasms or arteriovenous malformations) [27,28]. Due to the complete enzymatic degradation of ethanol, its toxic effects are limited to areas of high concentration and last only a short period. Li et al. [29] have employed ethanol injection to induce ONFH in a three-foot bearing canine model, and found subsequent resorption of necrotic trabeculae might weaken the structural properties of the femoral head *via* Micro-CT scanning. Reasonably, weight bearing plays an important role to result in the collapse of ONFH. Lameness, instability of the hip joint and subcapital fractures were not detected postoperatively. The experimental period was extended to 25 weeks to successfully mimic the progression of human disease from the early- to the end-stage of mechanical collapse.

Table 2. Percentage of fibrous tissue in the experimental groups and the control group ($\bar{x}\pm s$, CI).

| Group | Percentage of fibrosis | Confidence Interval |
|---------------|------------------------|---------------------|
| Control group | 4.93±2.410 | 4.23~5.63 |
| 4w | 39.61±9.474*,** | 33.59~45.63 |
| 8w | 46.35±6.979*,** | 41.93~50.77 |
| 12w | 49.64±6.909*,** | 45.26~54.02 |
| 25w | 57.05±7.588*,** | 52.23~61.87 |

* Difference between the experimental group and the control group was statistically significant ($p<0.0001$); ** difference between each experimental group was statistically significant ($p<0.05$), excluding the difference between the 8 week and the 12 week groups ($p=0.2576$).

Jones and Hungerford [30] have demonstrated that various etiological factors damage the microcirculation, which is a common pathological mechanism of femoral head osteonecrosis. Ethanol inactivates osteocytes directly and has toxic effects on adipocyte membranes that result in massive adipocyte swelling and a decline in the flow of the venous sinus blood. These processes directly destroy the microcirculation in the femoral head. Histopathology of the normal human femoral head has demonstrated that the percentage of osteocyte-filled lacunae correlates with age. Wong [31] has discovered that normal humans possess 92% viable osteocytes at 11–20 years of age, and the lost 8% might represent a sectioning artifact. Thereafter, osteocyte viability progressively decreases to 90% by age 30 and to 73–75% by age 40 or 50. Koo [32] considers the presence of more than 50% empty lacunae to indicate stage III (Arlet & Ficat) of ONFH. In the present study, the percentage of empty lacunae in the total control group was 2.53%, which was correlated to the natural preparation process or to normal phenomenon. However, the percentage of empty lacunae increased to 68.45% at postoperative week 8, which demonstrated that bone resorption played a predominant role. The percentage of empty lacunae and resorption and repair of bone debris occurred simultaneously at 12 and 25 weeks. Resorption might play a dominant role via the transformation of osteoprogenitor cells into osteocytes and the broadening of trabeculae in necrotic areas. Significant differences in the percentage of empty lacunae were determined between each experimental group. The percentage of empty lacunae increased at an early stage and then decreased via repair during the end stage of ONFH. These findings showed that the present animal model could successfully mimic the pathological changes observed in ONFH. In the present study, the percentage of empty lacunae was higher than that reported by Mannggold [33], most likely because the bilateral femoral heads were assessed, which resulted in an enhanced effect of weight bearing. The quantitative analysis of fibrous tissues revealed that repair of bone occurred after the induction of necrosis. Subsequently, the fibrous tissues increased in number and broadened, and they were transformed into woven bone in the necrotic area. The fibrous tissues were more mature at 25 weeks compared to 4 weeks, and this difference was statistically significant. This

result indicates that the bone repair reaction was extensive. Finally, the subchondral trabeculae were fractured due to bone resorption and mechanical weight bearing, and the resorption process predominated over the repair process. In the present study, only some stages among the pathological changes observed in ONFH were assessed; however, the pathological mechanism responsible for this condition remains unknown and requires further investigation.

CONCLUSIONS

Novel therapeutics for ONFH, including cytotherapy and physical therapy, has awaited satisfactory animal models for preclinical investigations [34,35]. The results of the current study are consistent with the ONFH changes observed in humans, and demonstrate the process of necrotic evolution. In this study, an animal model of ONFH based on histology is characterized, and this model might be suitable for the evaluation of novel therapeutic techniques in the treatment of ONFH.

Statement of conflict of interest

The authors declare that there are no conflicts of interest.

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