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# Warfarin reduces the incidence of osteonecrosis of the femoral head in spontaneously hypertensive rats

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**Abstract** In this study, we investigated the effects of warfarin potassium on the incidence of the femoral head osteonecrosis in spontaneously hypertensive rats (SHR). Twenty-four SHRs were divided into two equal groups, one given normal water (water group) and another provided with water containing warfarin (warfarin group). We compared the two groups histologically and observed the incidence of osteonecrosis. We also studied 17 Wistar Kyoto rats (WKY) to compare with SHR. Coagulation time, platelet count, and protein C activity were measured. Immunohistochemistry was also performed using endothelial nitric oxide synthetase (eNOS) antibody to investigate the function of endothelial cells. The incidence of osteonecrosis was significantly less in the warfarin group (10.5%) than in the water group (52.6%). Coagulation time was significantly longer in the warfarin group than the water group. Platelet count and protein C activity were not statistically different between the warfarin group and the water group. Results of immunohistochemistry revealed that endothelial cells in the femoral head were positive for eNOS in WKY but not in SHR. Our results indicated that warfarin reduced the incidence of femoral head necrosis in SHR.

**Key words** Femoral head necrosis · Spontaneously hypertensive rat (SHR) · Warfarin · Animal model

## Introduction

Osteonecrosis is established to afflict about 3000 new patients per year in Japan. Steroid hormone intake and heavy alcohol intake have been proposed as possible contributors to the disease process.[10,13,14] Especially, steroid-induced osteonecrosis comprises about 50% of the disease. Osteonecrosis is a debilitating disease affecting patients who have received corticosteroids for the treatment of such underlying disease as systemic lupus erythematosus, rheumatoid arthritis, bronchial asthma, and nephrotic syndrome and after organ transplant. High-dose administration of corticosteroids has been recently considered to be one of the major risk factors for osteonecrosis. Several mechanisms of corticosteroids induced osteonecrosis have been proposed: (1) steroid-induced hypercoagulable state[13]; (2) fatty emboli due to abnormal lipid metabolism[11]; and (3) increase in marrow fat cell size.[20] Several factors are associated with osteonecrosis in humans, but the precise cause of this condition remains obscure.

In a skeletal survey of spontaneously hypertensive rats (SHR), we incidentally observed widespread osteonecrosis, which occurred frequently in the epiphysis of the femoral head and noticed that the histological findings closely resembled those in humans; thus, we have used these rats as a model to investigate the etiology of osteonecrosis.[8,9] In this study, we considered osteonecrosis was related to hemostatic abnormality and studied the hemostatic state including endothelial cell function in SHR.

The aim of the present study was to evaluate the effect of warfarin on femoral head necrosis in SHR in an attempt to clarify the pathogenesis of this condition.

## Material and Methods

### *Animals*

Twenty-four male SHR/Izm rats (from Discase Model Cooperative Research Association, Kyoto, Japan) aged 5 weeks were used in this study. The SHR/Izm is a standard strain of SHR. All rats were housed under conditions of controlled temperature (24° ± 2°C), humidity (55% ± 2%), and artificial light from 0800 to 1800 each day at the Division of Comparative Medicine, Center for Frontier Life Sciences, Nagasaki University.

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All rats were fed a standard chow diet (F2; Funabashi Farm, Chiba, Japan) and tap water ad libitum. The rats were separated into two equal groups: one was given normal water (water group), and the other was provided with water containing warfarin potassium (Eisai, Tokyo, Japan) at 1.2 mg/l (warfarin group) from 6 weeks of age. The elected dose of warfarin was based on the reports from the surgical model of thrombosis by Hara et al.[7] The rats were killed under ethyl ether anesthesia from 15 to 17 weeks of age. In addition, 17 male Wistar Kyoto rats (WKY) were used to compare with SHR and were killed at the same time points. Systolic blood pressure (BP) was measured indirectly using an electrosphygmomanometer (TK 370A; Ynicom, Chiba, Japan) after prewarming the tail for 10 min at 38°C to dilate the caudal artery. Body weight and water intake were measured once a week, and daily intake of warfarin was calculated.

The experimental protocol was approved by the Guidelines for Animal Experimentation, Nagasaki University.

### *Histological examination*

For histological examination, the proximal femurs were fixed in 10% formalin solution and prepared for paraffin embedding after decalcification by ethylenediaminetetraacetic acid (EDTA). Subsequently, thin sections through the teres ligament were stained with hematoxylin and eosin and the histological changes were examined by light microscopy. We investigated the incidence of osteonecrosis of the femur histologically using the diagnostic criteria described by Ficat and Arlet.[3] Femoral head necrosis was also evaluated as a size ratio of necrotic area to epiphysis by measuring with the NIH Image.

### *Blood tests*

We collected blood samples via intracardiac aspiration to measure the number of platelets in SHR and WKY using an ACE counter FLC-240 (Fukuda Denshi, Tokyo, Japan). Protein C activity in the serum was measured in the laboratory of Mitsubishi Kagaku Bio-Chemical Laboratories (Tokyo, Japan). To examine the effect of warfarin, we performed the Thrombo-test (Nycomed Pharma, Oslo, Norway) to determine the coagulation time using Thrombo-Track (Sanko Junyaku, Tokyo, Japan). Blood warfarin concentrations were measured in the warfarin group by high performance liquid chromatography by Mitsubishi Kagaku Bio-Chemical Laboratories.

### *Immunohistochemistry*

Femoral heads were embedded in paraffin, cut into 5-μm serial sections, and stained with anti-endothelial nitric oxide synthetase (anti-eNOS) antibody using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Briefly, sections were treated with 0.3% hydrogen peroxide to inactive endogenous peroxidase and then with 10% nonimmune normal horse serum. After washing with 0.02 mol/l phosphate-buffered saline (PBS), the sections were incubated with primary antibody, eNOS antibody (PA3-031; Affinity BioReagents, Denver, CO, USA), for 45 min in a humid chamber at room temperature. The sections were washed thoroughly and incubated for 1 h with biotin-conjugated secondary antibody. After washing with PBS, avidin-biotinylated horseradish peroxidase was applied to each section for 30 min. After washing with PBS, peroxidase matrix, including 0.1% diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories) and 0.02% hydrogen peroxide, was dripped onto each section for 3 min. The sections were counterstained with hematoxylin.

### *Statistical analysis*

All data were expressed as mean ± standard error of mean. Differences between groups were examined for statistical significance using the Fisher's exact probability test and Mann—Whitney *U* test. *P* values less than 0.05 denoted a statistically significant difference.

## Results

### *Incidence of femoral head necrosis*

We considered the histological findings, including the dead trabeculae exhibiting empty lacunae (Fig. 1A,B) or tissue repair observed on the lateral side of epiphysis (Fig. 2A,B), to be osteonecrosis. We considered the histological findings including live trabecular bone, normal laminar structure, and marrow cavities filled with bone marrow components to be normal (Fig. 3A,B).

Femoral head necrosis was characteristically present in SHR from 15 to 17 weeks. The incidence of osteonecrosis in the water group was 52.6% (10 of the 19 femoral heads), while 10.5% (2 of the 19 femoral heads) in the warfarin group developed osteonecrosis. Statistical analysis showed a significantly higher incidence of osteonecrosis in the water group compared with the warfarin group.

In WKY, there was no incidence of femoral head necrosis. The area of osteonecrosis in the water group was 0.81% ± 0.48%, whereas 1.12% ± 0.79% in the

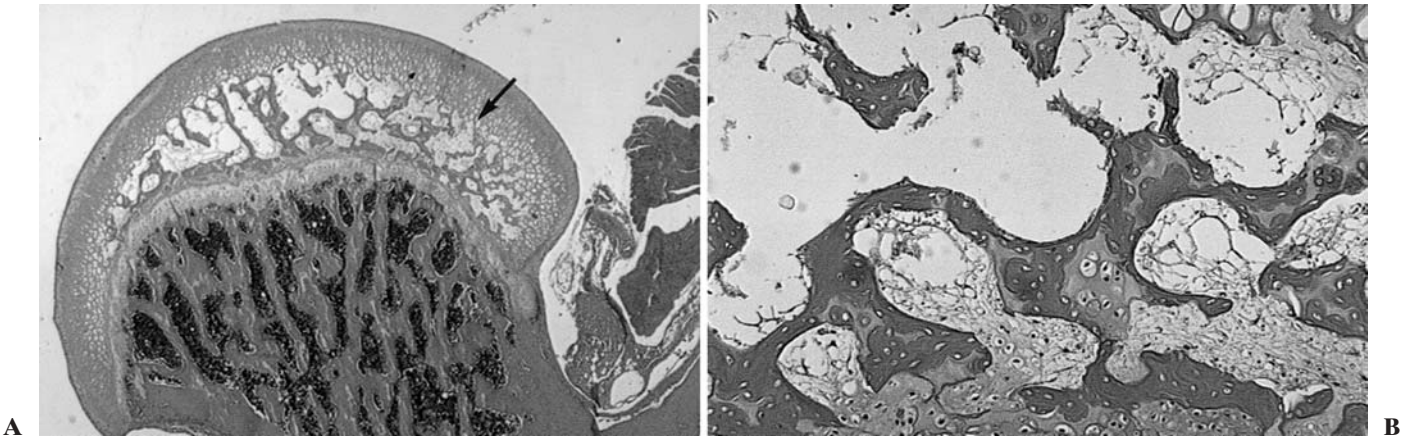

**Fig.1A,B.** Photomicrography of femoral head necrosis observed in spontaneously hypertensive rat (*SHR*) at 15 weeks of age. **A** Bone trabecular pattern is destroyed with wide cavities filled with necrotic debris. H&E, ×20. **B** Magnification of the section indicated in **A** (by *arrow*). There is total loss of osteocytes in the lacunae of trabecular bone. ×85

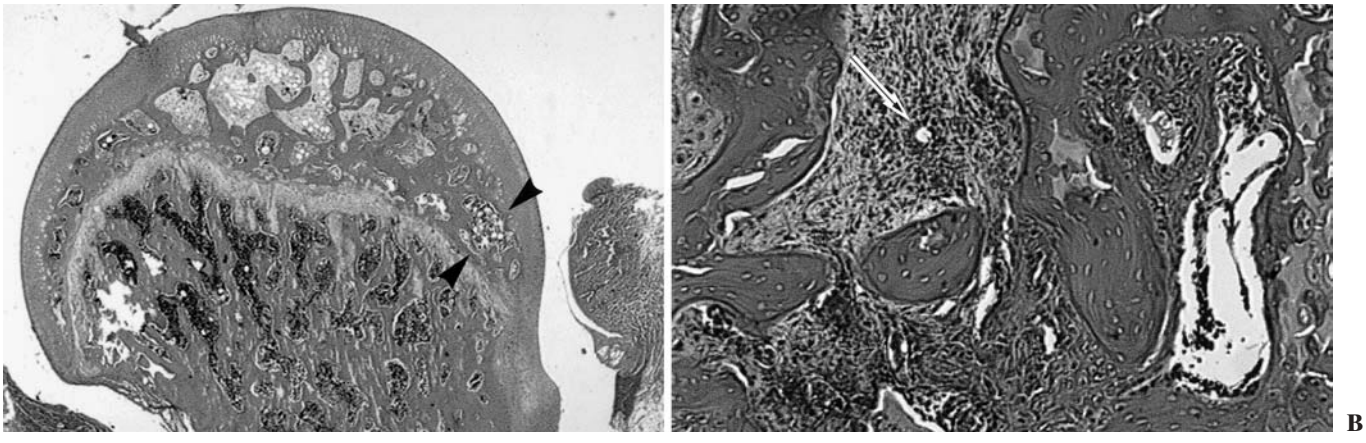

**Fig. 2A,B.** Photomicrography of femoral head necrosis observed in SHR at 16 weeks of age. **A** Tissue repair is observed on the lateral side of the epiphysis (*arrowheads*). H&E, ×20. **B** Necrotic marrow is replaced by reparative granulation tissue containing blood vessels (*white arrow*). ×85

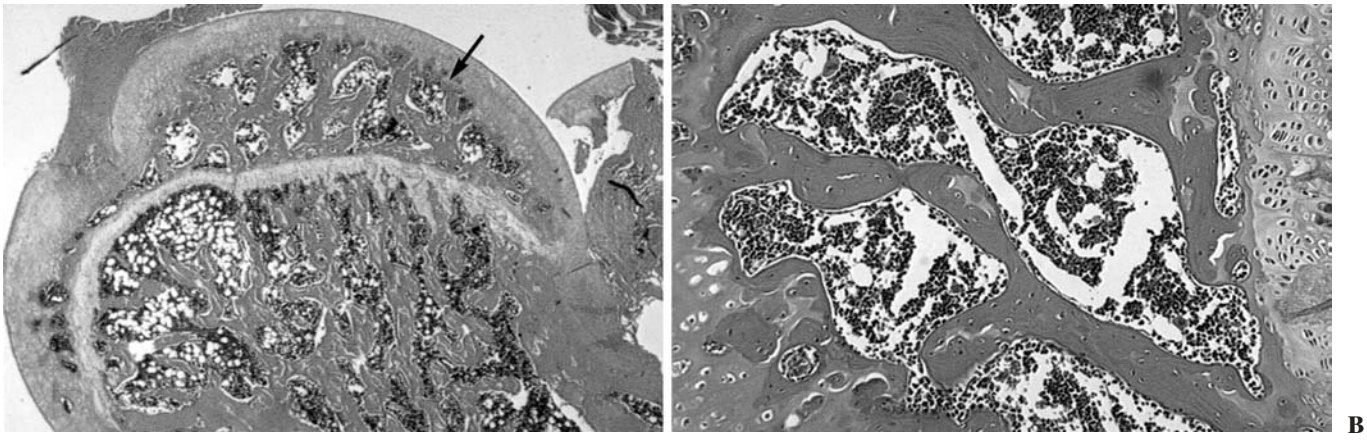

**Fig. 3A,B.** Photomicrography of femoral head necrosis observed in SHR administrated warfarin at 17 weeks of age. **A** Normal ossification in the epiphysis of femoral head. H&E, ×20. **B** Magnification of section indicated in **A** (by *arrow*). Normal femoral head exhibiting live trabecular bone, normal laminar construction, and marrow cavities filled with bone marrow components. ×85

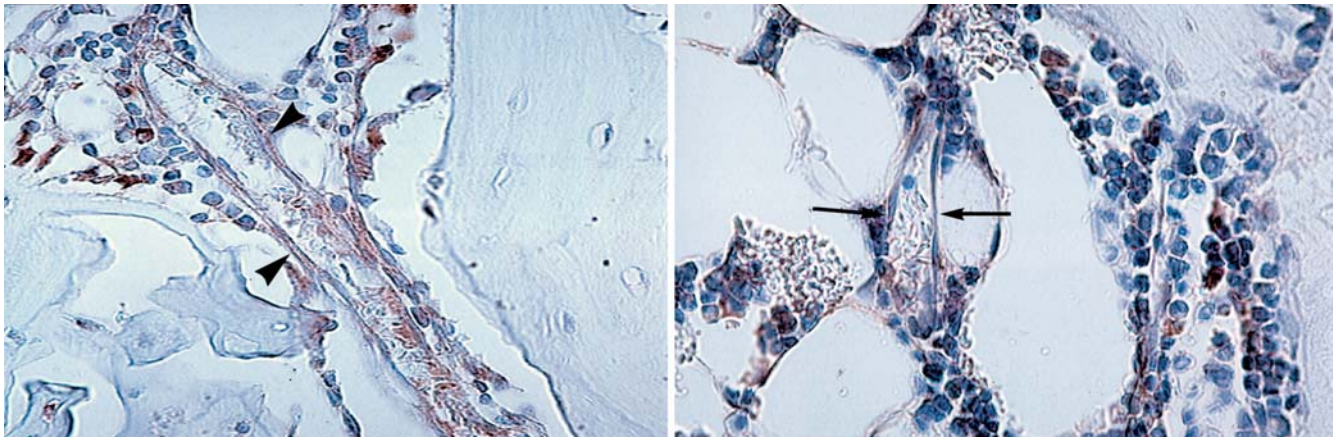

**Fig. 4A,B.** Blood vessels in the femoral head immunostained with endothelial nitric oxide synthase (*eNOS*) antibody. Endothelial cells stained positively in WKY at 15 weeks of age (**A**, *arrowheads*) but not SHR at 15 weeks of age in warfarin group (**B**, *arrows*). Counterstain, hematoxylin; ×400

warfarin group developed osteonecrosis. Statistical analysis did not show a difference in area osteonecrosis in water group compared with the warfarin group.

### *Systolic blood pressure (BP), body weight, and dose of warfarin*

At the beginning of the study, systolic BP of 5-week-old SHR was 128 ± 5 mmHg ($n = 12$) in the warfarin group and 132 ± 4 ($n = 12$) mmHg in the water group. Systolic BP in both groups increased gradually to more than 200 mmHg by the time of death. Rats in both groups gained body weight over the course of the experiment. However, there were no significant differences of the systolic BP and body weight values between the water and the warfarin groups throughout the study period. Daily warfarin intake was 0.28 ± 0.02 mg/kg (body weight) and seldom fluctuated throughout the duration of the experiment.

### *Blood evaluation*

At sacrifice, blood was collected and platelets were counted in SHR ($n = 15$) and WKY ($n = 13$). There were no significant differences at the platelet count between the water group (404 ± 38 × $10^4$/$mm^3$) and the warfarin group (400 ± 41 × $10^4$/$mm^3$). Protein C activity showed no difference in the water group (4.65% ± 5.66%) compared with the warfarin group (9.25% ± 9.00%). In contrast, the platelet count in SHR (402 ± 40 × $10^4$/$mm^3$) was significantly higher than that in the WKY (275 ± 48 × $10^4$/$mm^3$) ($P < 0.05$). As expected, the coagulation time was significantly prolonged in the warfarin group (58.2 ± 19.2 s, $n = 9$) compared to the water group (25.7 ± 2.4 s, $n = 6$)($P < 0.05$). The average warfarin concentration was 3.06 ± 0.92 ng/ml in the warfarin group ($n = 6$).

### *Immunohistochemistry*

We used four specimens for immunohistochemistry stain. Interestingly, immunohistochemical analysis of eNOS protein expression revealed eNOS positivity in the endothelial cells of WKY, but immunoreactivity against eNOS protein was not femoral in SHR, irrespective of warfarin administration (Fig. 4A,B).

## Discussion

Animal models of osteonecrosis have included the SHR, the steroid hormone-treated rabbit, and the immune complex rabbit.[6,8,9,16,18,19] Femoral head necrosis occurs spontaneously in SHR and is also repaired, with remnant empty lacunae of osteocytes and appositional bone formation. These characteristic osteonecrotic changes are comparable to those of femoral head necrosis seen in adult humans and appear mainly between the age of 15 and 17 weeks. Osteonecrosis occurs in the femoral epiphysis of the SHR, but in other animal models, it occurs mainly in the femoral metaphysis or diaphysis, which are different locations from the sites of femoral head necrosis that occurs in humans. Since 1988, the current authors have used SHR as a model of Perthes' disease to investigate the pathogenesis of this condition because the growth plate of rats is not closed. In this study, however, we used these rats as a model of femoral head necrosis. The major reasons why we considered SHR to be a model of osteonecrosis are as follows: (1) predilection of

osteonecrosis in SHR (15–17 weeks of age) is a reproductive age and equivalent to adult in humans; (2) osteonecrosis in SHR is a single event as well as in humans; and (3) osteonecrosis has often been found on normal growth plate.

Recent studies have indicated that femoral head necrosis could result from one or more pathological changes; these include arterial occlusion or constriction, congestion, and fat embolism.[11,17,18] Wang et al. suggested that increased fat cell volume in the bone marrow and increased bone marrow pressure produced a circulatory disturbance in the femoral head.[20] Jones suggested that intraosseous fat embolism triggered a thrombotic process of focal intravascular coagulation resulting in osteonecrosis.[11] On the other hand, some clinical case reports have suggested that osteonecrosis may be related to coagulation abnormalities.[1] Glueck et al. reported that heritable hypofibrinolysis and thrombophilia seemed to be the major pathoetiologies of osteonecrosis, and speculated that anticoagulation as a low molecular weight heparin might be a promising therapy for patients with osteonecrosis.[5] Nagasawa et al. researched patients with systemic lupus erythematosus (SLE) administrated corticosteroids for the hemostatic state and pointed out that lupus anticoagulant, which was often found in the plasma of patients with SLE, was associated with thrombosis and osteonecrosis.[14] Mont et al. studied risk factors for osteonecrosis in SLE and suggested anticoagulant therapy with warfarin might allow primary prevention of osteonecrosis.[13] Our study demonstrated that warfarin significantly reduced the incidence of the femoral head necrosis. Our results support similar findings reported by Glueck et al., who suggested that anticoagulation drugs could be a promising therapy for patients with osteonecrosis.[4]

Many factors impact upon thrombus formation including platelets, the coagulation–fibrinolysis system, and endothelial cell function. At the beginning of our study, we thought that SHR exhibited hypercoagulability. We speculated that the femoral head of SHR had endothelial cell dysfunction.[2,12] If endothelial cell damage occurred, abnormal blood coagulation and thrombi formation could follow, and any resulting osteonecrosis would therefore be found distal to the site of arterial occlusion.

Our immunohistochemical analysis suggested endothelial cell dysfunction, in particular resulting in less nitric oxide (NO) production, in SHR than WKY. NO has several anticoagulative actions, including dilatation of vessels, prevention of platelet aggregation, and inhibition of monocyte adherence to the endothelium. Our findings from eNOS immunostaining suggest lower production of NO in SHR, and thus these rats might be at a higher risk of thrombi formation. In this regard, Chou et al. used Western blot analysis to show that the basal activity and eNOS protein expression in aorta were significantly lower in SHR than that in WKY.[2] Further investigation is required to determine whether this endothelial cell dysfunction occurs primarily or is secondary to hypertension. Naito et al. reported that femoral head necrosis occurred in stroke-prone SHR despite treatment with antihypertensive agents, i.e., angiotensin-converting enzyme (ACE) inhibitors, even though other ischemic disorders such as brain and renal infarctions were prevented, indicating that femoral head necrosis does not represent a complication related to systemic hypertension.[15] Thus, although ACE inhibitors could lower the blood pressure of SHR, they do not reduce the incidence of osteonecrosis.

In conclusion, our results showed that administration of warfarin significantly reduced the incidence of femoral head necrosis in SHR, probably by minimizing circulation disturbances.

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