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Oxidative stress and vascular permeability in steroid-induced osteonecrosis model

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Abstract We focused on the role of oxidative stress in the pathogenesis of steroid-induced osteonecrosis (ON) and the possibility of preventing this condition by antioxidant administration. Methylprednisolone 4 mg/kg was injected only once into Japanese white rabbits. The involvement of oxidative stress and the presence/absence of bone circulatory impairment were investigated in groups of 10 rabbits killed at 3, 5, and 14 days each and in 10 rabbits administered the antioxidant glutathione. Reduced blood glutathione and lipid peroxide levels were determined biochemically, and the presence/absence of advanced glycation end-product expression was determined immunohistochemically. Vascular permeability in bone was confirmed by finding albumin leakage into the stroma. These blood biochemical and immunohistochemical studies clarified that the oxidative stress in this model developed 3–5 days after steroid administration. Elevated vascular permeability was observed in the 5- and 14-day groups. Hence, circulatory disturbance in bone was noted 5 days after steroid administration, coinciding with the onset of oxidative stress. The rate of ON development, which was 70% in the steroid-alone 14-day group, was significantly reduced to 0% in the steroid + antioxidant group. These results suggest the involvement of oxidative stress and vascular permeability in this steroid-induced ON model and the possibility of its prevention by suppression of oxidative stress.

Key words Steroid-induced osteonecrosis · Oxidative stress · Reduced glutathione · Lipid peroxide · Antioxidant

Introduction

The mechanism by which steroid-induced osteonecrosis (ON) develops has been variously attributed to direct injury to the vessels themselves (i.e., mediated by fat embolism,[6,12,13,24] enlargement of fat cells,[24] or vasculitis[25]) or to factors such as intramedullary hemorrhage, microvessel destruction,[20] or intravascular coagulation abnormalities.[11,29] Although steroid-induced ON is suggested to develop because of ischemia in the bone, the detailed mechanism is not understood. Therefore, it is important to understand the pathogenesis of steroid-induced ON due to steroid administration and to establish preventive methods to avoid this condition.

A number of animal experimental models aimed at elucidating the pathogenesis of steroid-induced ON have been performed.[14,15,28] The establishment of such models has raised hopes that the pathogenetic mechanisms underlying ON can be clarified; and based on the new knowledge obtained, better prophylactic measures can be devised.

On the other hand, it has become increasingly clear that oxidative stress is involved in the pathogenesis of a variety of diseases, including vascular injury and cell death.[2,8,17] Under conditions of oxidative stress, vascular and tissue injury occurs as a consequence of decreasing levels of reduced glutathione (GSH), which is necessary to maintain homeostasis,[4] increased lipid peroxide (LPO),[23] and protein modifications such as the appearance of advanced glycation end product (AGE).[9]

Apoptosis has recently been suggested to be one of the mechanisms underlying steroid-induced ON,[14] and tissue oxidation is known to induce apoptosis.[8] Furthermore, oxidative stress is thought to suppress the proliferation of osteoblasts[1,18] and to be cytotoxic to them.[5] For these reasons, our attention was drawn to oxidative stress, which is one of the factors inducing vascular injury and apoptosis, which are thought to play a role in the development of steroid-induced ON. We then undertook the present experiments.

In this study, injury to the blood circulation was examined by analyzing oxidative stress and vascular permeability in bone tissue in an ON model (rabbits given steroids). In addition, the involvement of oxidative

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stress in ON was examined by administering antioxidation agents to this model, along with their use for possible prevention of necrosis.

Materials and methods

Experiment 1: study of relation between steroid-induced development of oxidative stress and necrosis of bone and marrow

Adult female Japanese white rabbits (mean body weight 3.5 kg) were given a single injection of methylprednisolone (Upjohn, Tokyo, Japan) at a dose of 4 mg/kg body weight into the right gluteal muscle. Ten animals each were then killed 3, 5, and 14 days after steroid administration. They were designated the S3, S5, and S14 groups, respectively, and were compared to a control group of five rabbits that had been fed under the same conditions but had not undergone steroid administration.

Experiment 2: study of effects of antioxidants in steroid-induced ON model

On the day of administration and 7 days after administration of methylprednisolone (4 mg/kg into the right gluteal muscle), GSH 500 mg/kg was administered into the auricular veins of 10 rabbits, and the animals were killed 14 days later (G group).

At the end of each period, all rabbits were killed with an intravenous injection of pentobarbital sodium. As controls, five rabbits were injected with physiological saline for 7 days from the day of steroid administration. We adopted a methylprednisolone dose of 4 mg/kg for these studies, as it has been reported to reproducibly and reliably induce osteonecrosis.[15]

In experiments 1 and 2, immediately after death the bilateral femurs were isolated and fixed in 10% formalin for 1 week. The specimens were then decalcified in 10% EDTA. The specimens were embedded in paraffin and cut into 4-μm sections.

All protocols in this study were in accordance with the Animal Research Committee of Kanazawa Medical University.

The following items were examined in experiments 1 and 2.

Blood biochemistry

A sample of blood was drawn from the auricular vein before and 3, 5, 7, and 14 days after steroid administration. The blood concentrations of GSH and LPO were measured and the results expressed as the mean ± SD.

Immunohistochemistry

To examine the development of *oxidative stress* in bone, the femur was stained immunohistochemically with an anti-AGE monoclonal antibody (Trans Genic, Kumamoto, Japan). Briefly, after deparaffinization, the sections were treated with 0.3% H_2O_2 in methanol for 30 min at room temperature and with 0.1% trypsin for 15 min at 37°C. The sections were then reacted with anti-AGE antibody (400×) overnight at 4°C in a humidity chamber, followed by incubation with Dako EnVision/HRP system (Dako, Tokyo, Japan) for 30 min at room temperature. Sections were then treated with 3,3′-diaminobenzidine (DAB) for 5 min. Counterstaining was carried out with methyl green for 10 min.

To examine *vascular injury* in the femur, vascular permeability was evaluated using horseradish peroxidase (HRP) conjugated anti-albumin antibody (Cosmo Bio, Tokyo, Japan). Briefly, after deparaffinization, the sections were treated with 0.3% H_2O_2 in methanol for 1 h at room temperature and then reacted with HRP conjugated anti-albumin antibody (100×) for 1 h at room temperature in a humidity chamber. Sections were then treated with DAB for 4 min. Counterstaining was carried out with methyl green for 10 min.

Histopathology

Necrosis of bone and marrow tissues was examined in hematoxylin and eosin (H&E)-stained preparations with light microscopy. ON was judged "present" when there was necrosis of medullary hematopoietic cells or fat cells or there were empty lacunae or condensed nuclei in osteocytes. The development of osteonecrosis was judged to be "positive" when ON was identified in either isolated femur.[28] The rate of development of ON was calculated as the ratio of rabbits with ON/total number of rabbits used.

Statistical analysis

Comparison of GSH and LPO values before steroid administration and at 3, 5, 7, and 14 days thereafter were performed using a repeated-measures analysis of variance (ANOVA). The changes in GSH and LPO values between the S14 and G groups were compared using a two-way ANOVA. The rate of ON development was compared between the S14 and G groups using Yates's modified chi-squared test. Differences were considered significant at $P < 0.05$.

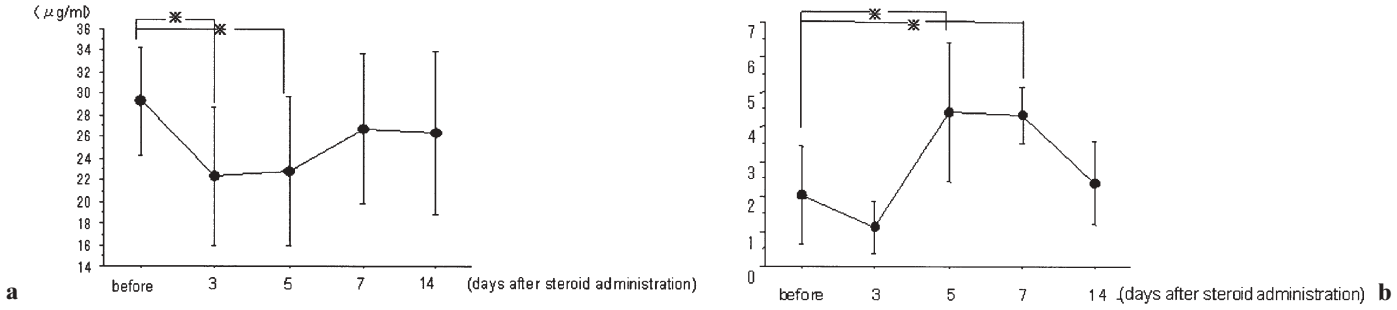

Fig. 1. Blood biochemical analysis during the development of oxidative stress in rabbits given a steroid. **a** Reduced glutathione was significantly decreased between 3 and 5 days after steroid administration (*$P < 0.05$), followed by a tendency to recover to a normal concentration 7–14 days after administration. **b** Lipid peroxide was significantly increased 5–7 days after steroid administration and recovered to almost the same level as that of the control on day 14 (*$P < 0.05$)

Results

Experiment 1: oxidative stress in the steroid-induced ON model

Blood biochemistry

The GSH was significantly decreased 3 days after steroid administration in the ON model animals and appeared to recover by 7–14 days after administration (Fig. 1). LPO was significantly increased 5–7 days after steroid administration and declined to almost the same level as that of the control group by day 14.

Immunohistochemistry

Development of oxidative stress in the femur. There was no positive reaction for AGE in the femur of the controls or the S3 group (Fig. 2). In contrast, in the S5 group an AGE-positive reaction was observed in the proximal femur. In the S14 group, clusters of AGE-positive cells were observed in and around necrotic areas. AGE-positive cells were observed scattered in the proximal femur and diaphysis but were clearly more sparse than in the peripheral femur around the necrotic areas.

Vascular permeability. There was no albumin leakage in the controls or the S3 group (Fig. 3). Albumin leakage was observed, however, in the stroma of the proximal femur in the S5 group, as demonstrated with AGE-specific antibodies. In the S14 group, stronger staining indicating leakage was observed more often in necrotic areas and in the stroma around areas of necrosis than in nonnecrotic areas.

Histopathology

There was no necrosis of the bone or bone marrow in the control, S3, or S5 groups (Fig. 4). ON was observed in 7 of 10 rabbits in the S14 group (70%).

Necrotic areas in the S14 group showed a clear boundary from the surrounding normal tissue. Empty lacunae were found in bone trabeculae, and the surrounding bone marrow tissue also showed necrotic changes (hematopoietic cell necrosis and fat cell necrosis).

Experiment 2: effect of oxidative stress inhibitors in the steroid-induced ON model

Blood biochemistry

There were no significant changes in GSH or LPO in the steroid-induced ON model animals, indicating protection against the development of oxidative stress. Compared to the S14 group in experiment 1, there were significant differences in the GSH level 3–5 days after steroid administration and in the LPO level 5–7 days after steroid administration (Fig. 5).

Immunohistochemistry

There was little AGE development in the bone (Fig. 6a) in the G group, as determined by immunohistochemical analysis. The development of oxidative stress in bone was also inhibited. There was little leakage of albumin into the stroma on albumin staining (Fig. 6b), similar to the results obtained with anti-AGE antibody.

Histopathology

In contrast to group S14, no rabbit in group G showed ON, representing a significant difference ($P < 0.01$), and in the latter group slight bone marrow necrosis was found in only two rabbits (Fig. 6c). There were no differences between the steroid-alone group and the five rabbits injected with saline alone with regard to changes in the GSH or LPO values or in the histological features of ON.

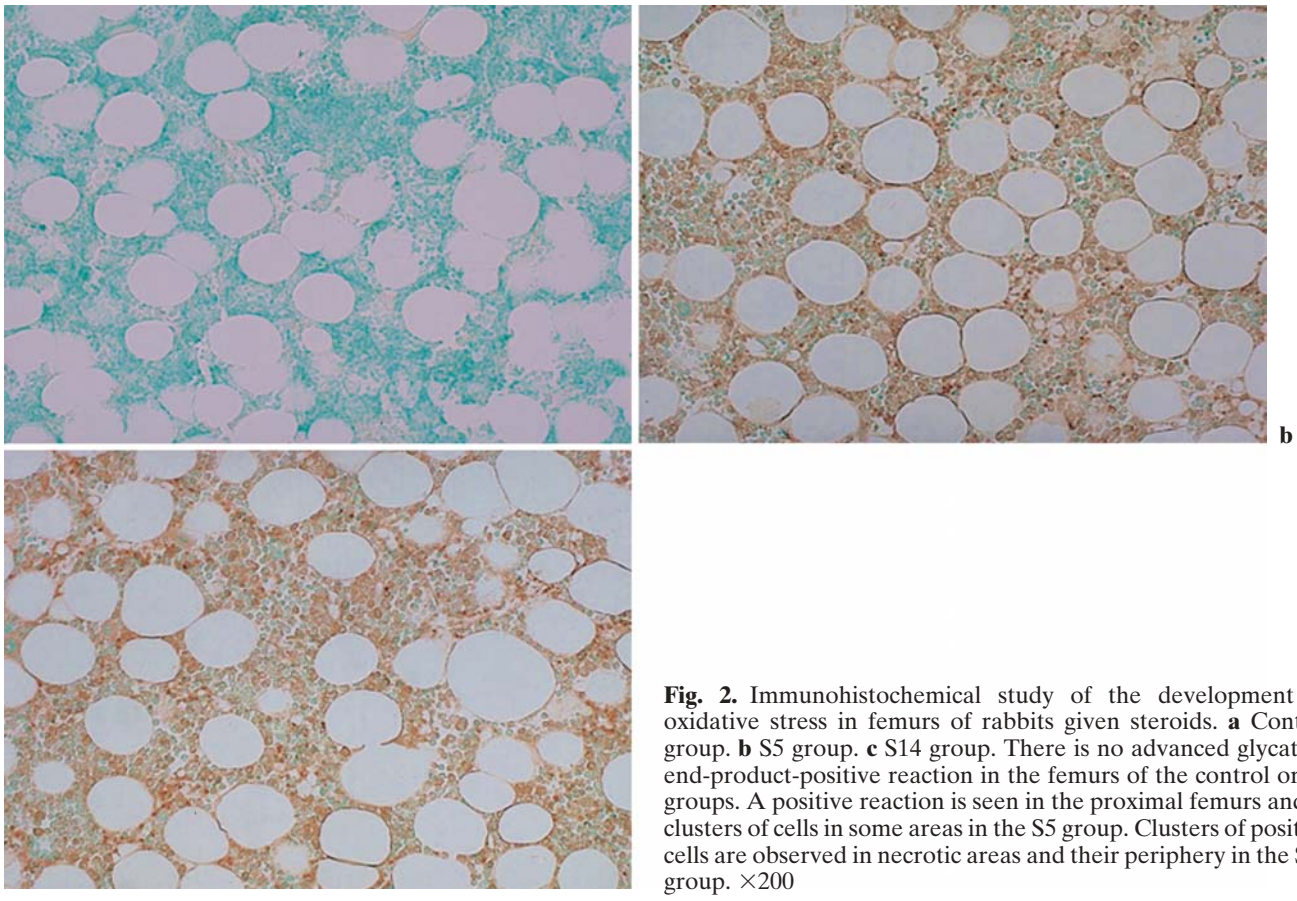

Fig. 2. Immunohistochemical study of the development of oxidative stress in femurs of rabbits given steroids. **a** Control group. **b** S5 group. **c** S14 group. There is no advanced glycation end-product-positive reaction in the femurs of the control or S3 groups. A positive reaction is seen in the proximal femurs and in clusters of cells in some areas in the S5 group. Clusters of positive cells are observed in necrotic areas and their periphery in the S14 group. ×200

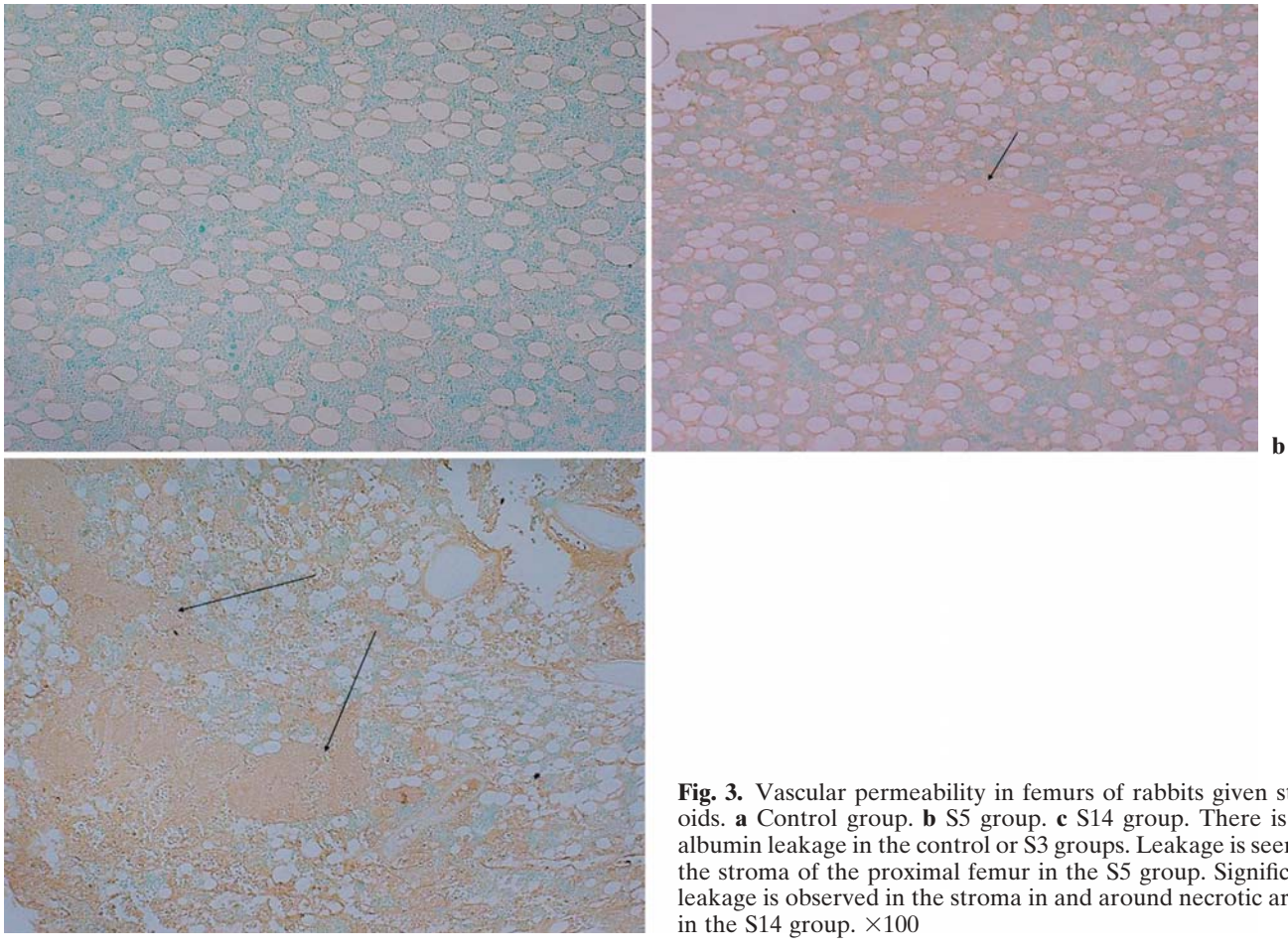

Fig. 3. Vascular permeability in femurs of rabbits given steroids. **a** Control group. **b** S5 group. **c** S14 group. There is no albumin leakage in the control or S3 groups. Leakage is seen in the stroma of the proximal femur in the S5 group. Significant leakage is observed in the stroma in and around necrotic areas in the S14 group. ×100

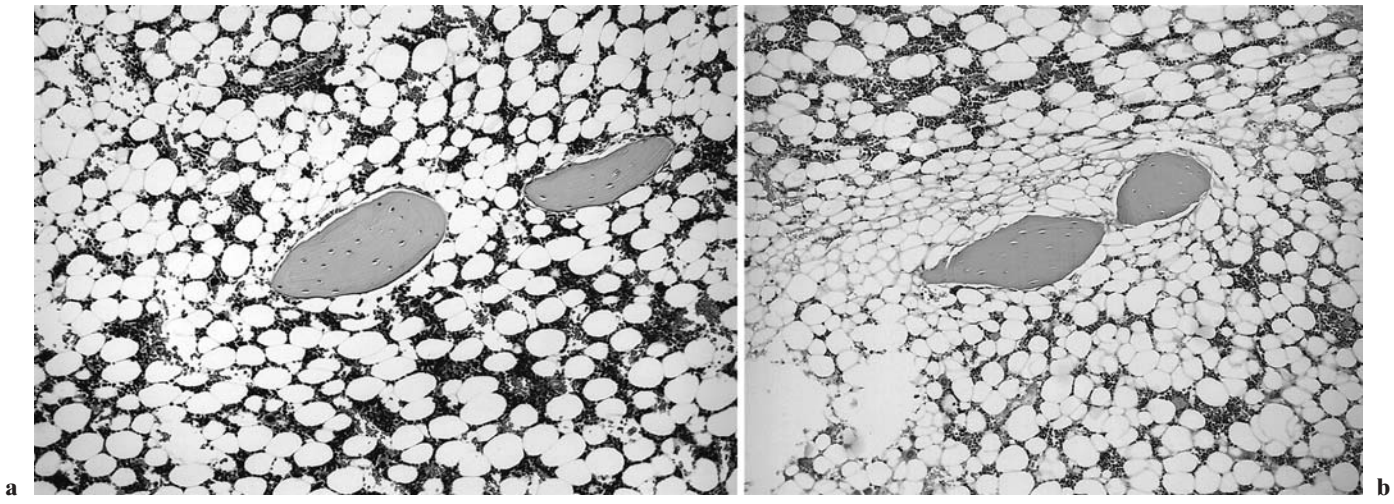

Fig. 4. Histopathological study of proximal femurs in rabbits given steroids. **a** Control group. **b** S14 group. There is no necrosis of bone or bone marrow in the control, S3, or S5 group. In the S14 group, osteocytes in the stained bone contain achromatic nuclei or empty lacunae, showing typical features of osteonecrosis. Medullary hematopoietic cells around the site of osteonecrosis are mixed with necrotic and degenerated cells, and necrotic fat cells are empty, having lost their cellular structures. ×100

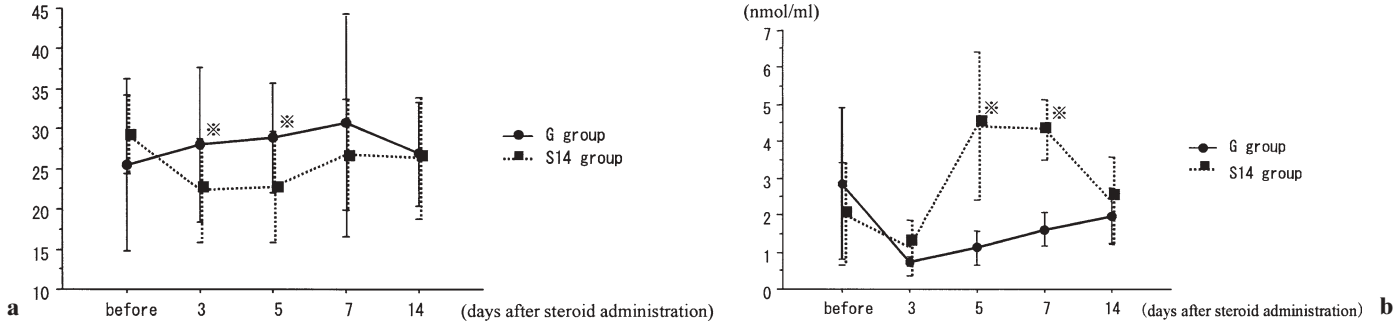

Fig. 5. Blood biochemistry in the group given steroid/glutathione concurrently and the group given steroid only. **a** Reduced glutathione. **b** Lipid peroxide. In the G group, reduced glutathione (GSH) and lipid peroxide (LPO) did not produce significant changes, indicating inhibition of oxidative stress. GSH and LPO showed significant differences 3–5 days and 5–7 days after steroid administration, respectively, compared to those in the S14 group ($*P < 0.05$)

Discussion

Steroid-induced ON is suggested to have a multifactorial pathogenesis, in which, in addition to circulatory derangements in bone and marrow, oxidative stress is also involved. In the present study we focused on changes in the blood levels of GSH and LPO because they are believed to reflect significantly the development of oxidative stress in the body.

Reduced glutathione is important for the homeostasis of cellular membranes, maintenance of the cytoskeleton, and metabolism of foreign substances.[19] It is suggested also that GSH stabilizes the lysosomal membrane and suppresses injury to the vascular endothelium and wall by inhibiting an increase of lipoperoxides,[3,26] maintaining a cellular oxidation-reduction balance,[10] and suppressing aggravation of metabolic dysfunction.[30]

Lipid peroxide, generated by hyperoxidation of lipids, is a biochemical indicator of tissue injury by active oxygens, which are suggested to be associated with vascular injury.[23] It has been reported that LPO markedly damages vascular endothelial cells,[21] smooth muscle cells, and macrophages;[22,27] it causes injury to the cell membrane by damaging the phospholipids constituting it.[7,16] It has been shown that oxidative stress plays an important role in the generation of AGE,[9] which was studied immunohistochemically in this study.

In our rabbit model, GSH and LPO showed significant changes 3–7 days after steroid administration, followed by a tendency to recover. AGE, as an indicator of the development of oxidative stress in the femur, was

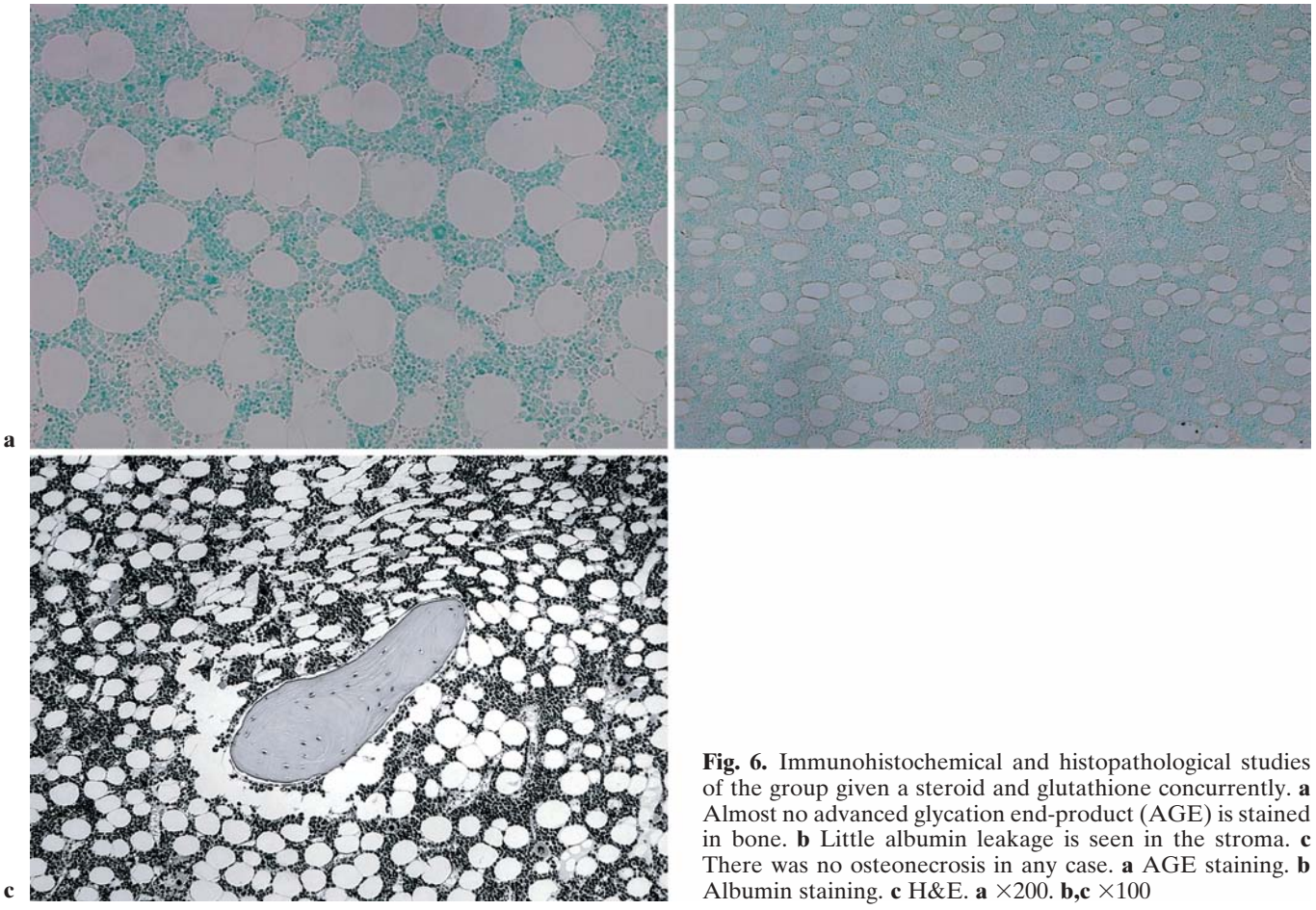

Fig. 6. Immunohistochemical and histopathological studies of the group given a steroid and glutathione concurrently. **a** Almost no advanced glycation end-product (AGE) is stained in bone. **b** Little albumin leakage is seen in the stroma. **c** There was no osteonecrosis in any case. **a** AGE staining. **b** Albumin staining. **c** H&E. **a** ×200. **b,c** ×100

not detected in the control or S3 groups but was identified in the S5 and S14 groups. These results suggest that steroid administration induced a condition of oxidative stress after only 3–5 days.

It was confirmed in the study on vascular permeability using an anti-albumin antibody that blood circulatory dysfunction, such as elevated vascular permeability and impaired hemostasis, occurred as early as 5 days after steroid administration, when oxidative stress was shown to occur. In this way, the early occurrence of these two events created conditions in which vascular and tissue injury are also likely to occur quite early after steroid administration.

The study examined the rate of development of ON after administration of an antioxidant, GSH. GSH is as an important factor for inhibiting the development of oxidative stress caused by steroid administration and therefore for preventing ON. It was confirmed that oxidative stress in bone was suppressed by GSH administration, as the indicators of oxidative stress, GSH and LPO in the blood, were significantly reduced; moreover, the appearance of AGE in the femur was inhibited by GSH administration in rabbits given the steroid. In addition, increased vascular permeability, measured by albumin staining, was suppressed, suggesting a relation between oxidative stress and vascular permeability. The fact that osteonecrosis development was significantly reduced in group G compared to group S14 implicates oxidative stress induced by steroid administration in the development of steroid-induced ON and suggests that the latter can be prevented by reducing oxidative stress.

With steroid administration, the level of glutathione needed to maintain a redox state in the body cannot be sustained, with associated lipid metabolic abnormalities inducing oxidative stress, increased LPO, and various protein modifications such as AGE. This vicious cycle leads to the accumulation of LPO and AGE, which are believed to cause tissue structural changes, vascular endothelial and wall injury, and increased vascular permeability, culminating in steroid-induced ON. The antioxidative agents, such as GSH, used in the present study are considered to act earlier in the oxidation process (i.e., at the stage of oxidative stress development), thereby stopping the entire subsequent vicious cycle from ever starting.

A limitation of the present study is that we did not perform an analysis at the gene level. This kind of more detailed analysis might provide further clarification of the mechanisms on which we focused here. We plan to undertake such a study in the near future.

The present results suggest that oxidative stress plays a crucial role in the development of steroid-induced ON in vivo. This is an important concept because it suggests the possibility of preventive approaches to block the development of oxidative stress. Because such antioxidants occur naturally in the body, it is anticipated that their use may be clinically feasible and effective in preventing the development of steroid-induced ON.

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