Edaravone, a novel free radical scavenger, prevents steroid-induced osteonecrosis in rabbits

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Abstract

Objective. To investigate the efficacy of edaravone, a novel free radical scavenger, on preventing steroid-induced osteonecrosis (ON) in a rabbit model.

Methods. Thirty-six New Zealand white rabbits were divided into control (C; n=6), steroid-administered (S; n=15) and edaravone-administered groups (E; n=15) after receiving an established protocol of steroid-induced ON. Before and after steroid administration, plasma levels of reduced glutathione (GSH) and lipid peroxidation (LPO) were measured for oxidative stress. Two weeks later bilateral proximal femurs were dissected for micro-CT-based micro-angiography, and the presence or absence of ON and intra-vascular thrombi were examined histopathologically. Immunohistochemical examination of oxidative injury in bone tissue was conducted using the anti-8-hydroxy-2'-deoxyguanosine and anti-malondialdehyde mAbs.

Results. The incidence of ON in the E group (20%) was significantly lower than in the S group (73%). Three to five days after steroid administration, the plasma GSH level was significantly higher and LPO level was significantly lower in the E group than the S group. Compared with the S group, there were significantly more small-sized perfusion vessels and fewer large-sized dilated vessels in the E group. Thrombosis incidence was significantly lower in the E group than the S group. Intraosseous vessels and haematopoietic cells that sustained oxidative injury were significantly fewer in the E group than the S group.

Conclusion. Edaravone exerted beneficial effects on reducing incidence of steroid-induced ON by suppressing the accumulation of lipid peroxidative products and oxidative DNA damage in endothelial cells and haematopoietic cells.

Key words: steroid-induced osteonecrosis, oxidative stress, edaravone, lipid peroxidation, DNA damage.

Introduction

Steroids are widely used for the treatment of chronic autoimmune diseases such as SLE, in acute medical situations such as traumatic spinal cord injury and brain injury, and for immunosuppression after organ transplants [1–3]. Steroid-induced osteonecrosis (ON) of the femoral head has been reported to occur frequently in patients receiving high-dose steroid treatment for these underlying diseases [4]. As steroid-induced ON tends to be popular among young patients and the surgical prognosis is generally poor [5], it is of great importance to clarify the pathophysiology of steroid-induced ON and develop a strategy to prevent its occurrence.

Despite the strong association of steroid use with ON in clinic, the underlying mechanisms remain unclear. Some studies have suggested thrombotic and fibrinolytic disorders, which predispose to both hypercoagulation and hypofibrinolysis, as possible aetiologic causes of steroid-induced ON [6]. Extravascular lipid deposition and subsequent elevated intraosseous pressure were also considered as an important contributor to the development of steroid-induced ON [7]. Vascular endothelial dysfunction, which may amplify hypercoagulability, has also been implicated in the pathophysiology of steroid-induced ON [8]. Recently, oxidative stress, which is implicated in a variety of pathological conditions including vascular injury and cell apoptosis [9, 10], has been
reported to play an important role in the development of ON [11]. In a recent study, the pro-oxidant buthionine sulfoximine successfully induced ON in rats [12]. Furthermore, the presence of oxidation injury in bone was demonstrated by the detection of strong immunohistochemical staining for oxidative stress in bone marrow vessels, myelocytes and adipocytes in animal models of steroid-induced ON [13, 14]. Additionally, studies have shown that steroid excess can induce the overproduction of reactive oxygen species (ROS) [15].

Thus, we assume that antioxidative substances could be used to prevent steroid-induced ON by alleviating oxidative injury caused by steroid use. Several antioxidants, such as vitamin E and reduced glutathione (GSH), have been demonstrated to be effective in preventing steroid-induced ON [11, 14]. Edaravone, a strong and novel free radical scavenger, is clinically used mainly for the treatment of ischaemia reperfusion injury after acute cerebral infarction and myocardial infarction [16]. Edaravone exhibits promising antioxidative functions, including enhancement of prostacyclin production, inhibition of hydroxyl radical dependent and independent lipid peroxidation (LPO), and quenching of active oxygen [17]. It exerts inhibitory effects on both water-soluble and lipid-soluble peroxyl radical-induced peroxidation, similar to the combined effects of vitamins C and E [18]. Accordingly, we hypothesized that edaravone might exert beneficial effects on preventing steroid-induced ON development. The purpose of this study was to test the hypothesis in an established rabbit model of steroid-induced ON.

Materials and methods

Animal, group and treatment

Thirty-six male 28-week-old healthy, pathogen-free adult New Zealand white rabbits (mean body weight 2.8 kg) were chosen for the experiment. The animals received a standard laboratory diet and water ad libitum. Fifteen rabbits were injected once into the right gluteal muscle with 20 mg/kg of the steroid (methylprednisolone, Pfizer Pharmaceutical, Shanghai, China) alone (C group). Fifteen other rabbits received intra-auricular venous injections of 3 mg/kg/day of edaravone (Simcere, Nanjing, China) for seven consecutive days (E group) in addition to the steroid, starting from the day of steroid administration. The intraosseous vascular structure of the proximal femur was determined based on the diffuse presence of reactive oxygen species (ROS) [15].

Histology

Two weeks after steroid administration, the remaining 18 rabbits in the S and E groups were also sacrificed with an excessive i.v. dose of sodium pentobarbital. The bilateral femurs were immediately harvested and fixed in 10% buffered formalin for 1 week, and then decalcified with 10% EDTA for 3 weeks. After decalcification, all femurs including the ones undergoing micro-angiography were embedded in paraffin. Four-micrometre thick coronal sections of the femurs were prepared and stained with haematoxylin and eosin.

The presence or absence of ON was determined in four sections taken from the proximal ends of bilateral femurs of each rabbit by light microscopy. A positive diagnosis of ON was determined based on the diffuse presence of empty lacunae or condensed nuclei of osteocytes within the bone trabeculae, accompanied by surrounding bone marrow cell necrosis or fat cell necrosis [22]. The development of ON was judged to be positive when ON was identified in either isolated femur. The ON incidence was calculated as the ratio of the number of rabbits with ON/total number of rabbits. Intravascular thrombus was detected by examining the entire area of four sections taken from the proximal ends of bilateral femurs of each rabbit, and thrombus-positive vessels were counted based on the presence of thrombus within the marrow vessels.

Immunohistochemistry

For the detection of DNA oxidative injury and LPO in bone, femurs were stained immunohistochemically with anti-8-hydroxy-2’-deoxyguanosine (8-OHdG) mAb (N45.1, Japan Institute for the Control of Aging, Shizuoka, Japan) and anti-malondialdehyde (MDA) mAb (clone 1F83, Japan Institute for the Control of Aging, Shizuoka, Japan) respectively [13, 19].

Briefly, after deparaffinization, sections were treated with 3% H2O2 for 10 min, rinsed in PBS (pH 7.6), and
pretreated with 3% non-immune animal serum in PBS for 30 min at room temperature. Sections were then reacted with clone 1F83 or N45.1 mAb overnight at 4°C. Sections were stained using the avidin–biotin complex method, treated with 3,3’-diaminobenzidine (DAB), and counterstained with haematoxylin.

Two sets of immunohistological data (8-OHdG and MDA) were quantified using NIS-Elements image viewer software (Nikon, Tokyo, Japan). Oxidative injury was evaluated by calculating the positive ratio (%PR, the percentage of positive cells/total cells) in five randomly selected visual fields (×200 magnification). The 8-OHdG positive marrow vessels were also counted in five randomly selected visual fields (×100 magnification).

Statistics
Categorical data, i.e. incidence of ON, were analysed using Fisher’s exact probability test. Numerical data in each group were expressed as mean (S.D.). Simple comparisons of numerical data were performed using Student’s t-test. Comparison of GSH and LPO values before and at 3, 5, 7 and 14 days after steroid administration in each group were performed using a repeated-measures analysis of variance (ANOVA). The changes in GSH and LPO values between the S and E groups were compared using a two-way ANOVA.

Results
Histological results
The ON incidence was 73% (11/15) in the S group and 20% (3/15) in the E group (Figs 1 and 2). Fisher’s exact probability test showed that the ON incidence in the E group was significantly lower than that of the S group (P < 0.01). No rabbit was diagnosed with ON in the C group.

Histologically, the number of thrombus-positive vessels was 4.13 (1.46) in the S group and 2.27 (1.33) in the E group (P < 0.001). The increased thrombotic vessel number in the S group was obviously attenuated in the E group (Figs 1 and 2). No thrombus-positive vessel was found in the C group.

Haematological findings
GSH levels were significantly decreased 3–7 days after steroid administration (P < 0.01) in the S group and

Statistical analysis of MDA %PR, 8-OHdG %PR and the distribution of different size micro-CT-based angiographic structural units of each group were performed by one-way ANOVA. All statistical analysis was performed using SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Statistical significance for comparison was set at P < 0.05.

Fig. 1 Histological features of both normal and necrotic bone.

(A) Normal bone tissue with intact cellular structures. (B) Typical osteonecrotic lesion: empty lacucae or condensed nuclei of osteocytes within the bone trabeculae (white arrow). Medullary haematopoietic cells around the site of ON were mixed with necrotic and degenerated cells, and necrotic fat cells lost their cellular structures (black arrow). (C) In the S group, thrombi (black block arrow) were found in some marrow vessels. (D) In the E group, angiographic substance (white block arrow) was detected in many marrow vessels. Stain: haematoxylin and eosin; magnification: ×200 (A, B), ×400 (C, D).
appeared to recover by 7–14 days after administration. LPO levels were significantly increased 5 days after steroid administration ($P < 0.01$) in the S group and returned to baseline thereafter.

There was significant difference in GSH levels 3–5 days after steroid administration ($P < 0.01$) and in LPO levels 5 days after steroid administration ($P < 0.01$) between the S and E groups. In particular, both decreases in GSH and increases in LPO in the S group were significantly attenuated in the E group (Fig. 3A and B).

**Immunohistochemistry**

Clusters of haematopoietic cells and many vessels showed strong immunoreactivity with anti-8-OHdG mAb in the S group, whereas there were only a few, sporadic 8-OHdG-positive cells and vessels in the E and C groups (Fig. 4A and B). Specifically, 8-OHdG %PR was 15.99 (3.50) in the S group, 8.91 (2.71) in the E group and 3.67 (1.84) in the C group, representing a significantly higher positive ratio in the S group compared with the E and C groups ($P < 0.01$ for both S group vs E group and S group vs C group). There were significantly more positive cells in the E group than the C group ($P < 0.01$) (Fig. 5C).

**Micro-CT-based evaluation of vascular network**

By the micro-CT imaging software, vessels were classified into seven groups according to the vessel diameter: Grade I ($37–110\,\mu m$), grade II ($111–183\,\mu m$), grade III ($184–256\,\mu m$), grade IV ($257–329\,\mu m$), grade V ($330–402\,\mu m$), grade VI ($403–475\,\mu m$) and grade VII ($>476\,\mu m$). Bone marrow vessels of the proximal femurs (above the lesser trochanter) with different diameters were reconstructed in 3D for presentation based on mapping colour-coded scales (Fig. 6A and B). As shown in Fig. 6C, representative histograms were compiled to show the vessel frequency in different groups and grade II ($111–183\,\mu m$) vessels were dominant in the proximal femur vascular network. As compared with the S group, the angiograms in the E and C groups showed a significantly higher frequency of small-sized vessels (grade II) ($P < 0.05$ for S group vs E group, $P < 0.01$ for S group vs C group), and lower frequency of large-sized vessels (grade VII) ($P < 0.01$ for both S group vs E group and S group vs C group). Although the frequency of small-sized vessels (grade II) was lower in the E group than the C group, there was no significant difference between the two groups ($P > 0.05$). Similarly, no significant difference was found in the frequency of large-sized vessels (grade VII) between the E and C groups ($P > 0.05$). No significant difference was observed in the frequency of medium-sized vessels (grades III and VI) between the three groups ($P > 0.05$).

Based on the distribution of different size vessels, the vascular structure pattern of the proximal femur in the E and C groups was almost the same.
Both in vitro and in vivo data have recently shown that steroid-associated oxidative injury exerts a crucial role in the development of ON, and several antioxidants have been reported to effectively suppress the development of steroid-induced ON [11, 13, 14, 15, 19, 20, 23, 24]. Soon after steroid administration, attenuation of protective antioxidative mechanisms occurs, leading to tissue peroxidation and protein modifications in bone tissues [19]. Vascular endothelial dysfunction, which contributes to local intravascular thrombosis and circulatory disturbances [25], occurs concomitantly with oxidative injury in ON [23]. In addition to the vascular endothelial damage, haematopoietic cells that sustain oxidative injury from steroid treatment also contribute to the development of steroid-induced ON. Kabata et al. [26] have reported the role of apoptosis in steroid-induced ON and haematopoietic cell apoptosis occurring in the early stages of steroid-induced ON.

Edaravone, a novel free radical scavenger, plays an extremely important role in the suppression of the negative oxidative stress cycle against both hydroxyl radical and iron-dependent LPO [27, 28]. In the present study, the administration of edaravone significantly reduced the incidence of steroid-induced ON in an established rabbit model to 20% as compared with 73% in the steroid-treated only group. The beneficial effect of edaravone on

**Discussion**

(A) Significantly decreased GSH from baseline in the S group was attenuated in the E group. (B) Significantly increased LPO from baseline in the S group was attenuated in the E group. **P < 0.01 for comparison with the S group; #P < 0.01 for comparison with baseline.**

![Figure 3 Haematological data analysis.](A) Significantly decreased GSH from baseline in the S group was attenuated in the E group. (B) Significantly increased LPO from baseline in the S group was attenuated in the E group. **P < 0.01 for comparison with the S group; #P < 0.01 for comparison with baseline.
Preventive efficacy of edaravone against osteonecrosis

Preventing steroid-induced ON was demonstrated in two aspects: (i) protecting the structure-function of intraoss
eous vasculature system and (ii) protecting the haematopoietic cells from oxidative injury by steroid treatment.

The plasma levels of GSH and LPO, two important biochemical markers of oxidative stress, were measured to

assess the redox state in rabbits. GSH constitutes the major intracellular antioxidant defence and has been shown to be a scavenger of a wide range of reactive species including ROS and reactive nitrogen species [29]. LPO is a biochemical indicator of tissue injury by ROS. It is generated by hyperoxidation of lipids and could

Fig. 4 Immunohistochemical study of the development of oxidative stress in proximal femurs of rabbits.

(A) In the S group, clusters of positive haematopoietic cells and many positive vessels were detected. (B) In the E group, only a few sporadic positive cells were seen. (C) In the S group, many positive haematopoietic cells were detected. (D) In the E group, only a few positive haematopoietic cells were seen. Stain: anti-8-OHdG mAb (N45.1) (A, B) and anti-MDA mAb (clone 1F83) (C, D); magnification: ×200.

Fig. 5 Immunohistochemical data analysis.

(A) A lower 8-OHdG %PR was found in the E and C groups than the S group (P < 0.01). (B) 8-OHdG positive vessel number was significantly higher in the S group than the E and C groups (P < 0.01). (C) A lower MDA %PR was found in the E and C groups than the S group (P < 0.01). **P < 0.01.
severely disturb the redox balance, triggering cell apoptosis [30–32]. We have shown that shortly after steroid administration, a significant decrease in GSH levels and increase in LPO levels was detected, which is consistent with findings by Ichiseki et al. [11]. Additionally, we further demonstrated that the decrease of GSH and increase of LPO in blood could be significantly attenuated by edaravone administration. This implied a protective effect of edaravone on oxidative injury caused by steroid. This is not surprising given that edaravone has previously been shown to prevent oxidative damage in a variety of oxidation-related diseases [16].

Changes in the intraosseous vasculature have also been documented during the development of ON. Atsumi and his colleagues [33] have found increased peripheral vessels with greater diameters in the proximal and weight-bearing areas of the femoral heads with early-stage ON as compared with normal femoral heads. Using a high resolution micro-CT-based angiography, we examined the intraosseous vascular network structure in the proximal femur following steroid administration and following treatment with edaravone. Two weeks after steroid administration, a significantly lower frequency of small-sized perfused vessels (111–183 μm) and higher frequency of large-sized vessels (≥476 μm) were observed in the steroid-treated only group. Similar changes in small-sized perfused vessels in a steroid-induced ON rabbit model were also observed by Zhang [34].

Fig. 6 Representative 3D intraosseous angiograms of the proximal femur from micro-CT-based angiography and angiographic data analysis.
et al. [34]. Previous studies have suggested damage to functional microvessels or capillary rarefaction as possible underlying mechanisms of ON [35, 36]. The increase in frequency of large-sized vessels observed following steroid administration could be attributed to the dilatation of normal vessels owing to blockage of distal small-sized perfused vessels by thrombi.

The decrease in frequency of small-sized perfused vessels in proximal femurs after steroid administration was further testified by histological findings of increased thrombotic vessels, which is consistent with previous reports in the literature [34, 37]. On the other hand, with edaravone treatment following steroid administration, both the decrease in small-sized perfused vessels and increase of intravascular thrombus formation was significantly ameliorated, indicating a protective effect of edaravone on the local arterial supply.

Endothelium injury has been widely recognized to be an important initial contributor to local hypercoagulability and intravascular thrombus formation [25]. Vascular endothelial cells that are constantly exposed to blood flow and risk factors are susceptible to injury by oxidative stress [38]. Haematopoietic cell injury was also demonstrated to occur in the early stage of steroid-induced ON [26]. Immunohistochemical staining of 8-OHdG, which is produced when DNA sustains oxidative injury due to ROS and is considered an important marker of oxidative injury [39], demonstrated that bone marrow vessels and surrounding haematopoietic cells sustained extensive oxidative injury owing to steroid administration, compared with the control group. Additionally, staining for MDA, one of several low-molecular-weight end products formed following decomposition of certain primary and secondary LPO products [40], was also markedly increased following steroid administration. Treatment with edaravone considerably reduced steroid-induced DNA oxidative injury and LPO stress to endothelial and haematopoietic cells. Studies by Xi et al. [41] in a rat model also demonstrated that edaravone could attenuate oxidative stress-induced endothelial damage, further reinforcing our findings. Together, our data suggest that vascular and haematopoietic cell damage as a result of steroid-induced DNA oxidative and lipid peroxidative injury can be successfully attenuated by edaravone administration, which was in accordance with the investigation of edaravone in other oxidation-related diseases [16, 42].

Collectively, our findings suggested that oxidative stress was closely involved in the development of steroid-induced ON. Our experiment also demonstrated that edaravone exerted noticeably beneficial effects on preventing the development of ON by inhibiting oxidative injury to vasculature and haematopoietic cells in bone marrow. As a novel antioxidant, edaravone is currently mainly used for the treatment of patients with acute-stage cerebral infarction in clinic [27]. In addition to the neuroprotective effects, edaravone has also been shown to prevent oxidative damage to various extracerebral organs, such as acute myocardial infarction [28]. Edaravone has been proven to be well tolerated among patients, with low rate of side-effects [16]. Given its wide clinical utilization and our positive findings, administration of edaravone may be an efficient and safe strategy for the prevention of early-stage, steroid-induced ON. However, the cause of ON is multifactorial and other mechanisms implicated in the development of ON including disorders of lipid metabolism, inhibition of angiogenesis, and abnormalities in the coagulation and fibrinolytic system, need further investigations [34, 43].

A limitation of the present study is the single dosage of edaravone treatment. Dosing effect needs to be further explored to determine the optimal dose on reducing incidence of steroid-induced ON. Moreover, the 2-week duration for observation designed in our study could be extended to determine the long-term efficacy of edaravone treatment.

In conclusion, the findings of the present study demonstrate that oxidative stress is an important contributor to the development of steroid-induced ON. Edaravone, a novel and potent free radical scavenger, could significantly reduce the incidence of steroid-induced ON by suppressing the accumulation of lipid peroxidative products and oxidative DNA damage in vascular endothelial and haematopoietic cells, thereby maintaining the normal redox state and intraosseous vascular integrity within the bone marrow.

**Rheumatology key messages**

- Oxidative stress contributes to the development of steroid-induced ON in the rabbit model.
- Edaravone exerts beneficial effects on reducing incidence of steroid-induced ON by suppressing oxidative stress in rabbits.

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