EXPERIMENTAL STUDY

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Preventive effects of coenzyme Q_{10} (CoQ_{10}) on steroid-induced osteonecrosis in rats

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Objective: The aim of this study was to examine the role of coenzyme Q_{10} (CoQ_{10}) in the prevention of steroid-induced osteonecrosis of the femoral head (ONFH) in rats.

Methods: The study included 20 Sprague-Dawley rats injected once with 20 mg/kg of methylprednisolone acetate into the right gluteus medius muscle to induce osteonecrosis. Animals were divided into two equal groups; Group 1 received no prophylaxis (control group) and the Group 2 received CoQ_{10}. Hematological examinations were performed before steroid injection (0 weeks) and at 4 weeks after steroid injection. Femoral heads were examined histologically to evaluate osteonecrosis.

Results: Changes in blood glutathione (GSH) and malondialdehyde (MDA) concentrations were less significant in the CoQ_{10} group. The incidence of histologic changes consistent with early osteonecrosis was lower in the CoQ_{10} group (2 of 10; 20%) than the control group (7 of 10; 70%).

Conclusion: Coenzyme Q_{10} may be useful as a preventing agent in steroid-induced ONFH. Inhibited oxidative stress is a possible mechanism for this effect.

Key words: Coenzyme Q_{10}; glutathione; malondialdehyde; steroid-induced osteonecrosis.

Osteonecrosis of the femoral head (ONFH) is described as the destruction of the trabecular bone and bone marrow, resulting in serious morbidity and disability of the hip joint. Osteonecrosis of the femoral head is the most common orthopedic complication of corticosteroids leading to total hip arthroplasty in young adults.[1] Prevention of ONFH after corticosteroid administration is an ideal strategy for the treatment of this condition. For this purpose, different therapies such as anti-coagulant agents, anti-lipid agents and antioxidant agents have been suggested.[2-4] Even though the development of steroid-induced osteonecrosis is attributed to ischemia in the bone, the exhaustive mechanism of the corticosteroid administration on the development of osteonecrosis remains unclear.[5] Recently, *in vivo* oxidative stress has been reported to play a role in the pathogenesis of steroid-induced osteonecrosis.[6,7]

Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble, vitamin-like substance and a potent antioxidant that plays a role in membrane stabilization. It can be used in the treatment of a variety of disorders, such as heart diseases, infertility, cancer, and nervous system diseases.[8] CoQ_{10} is involved in prevention of oxidative stress-induced apoptosis in neuronal cells.[9] To our knowledge, no studies on

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the use of CoQ_{10} in ONFH have been reported.

The aim of this study was to examine the role of CoQ_{10} in the prevention of steroid-induced ONFH in rats. We hypothesized that CoQ_{10} could have a protective effect against the steroid-induced ONFH.

Materials and methods

The current study was reviewed and sanctioned by the Animal Research Ethical Committee and animal experiments were conducted in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (revised 1985). The study included 20 male Sprague-Dawley rats weighing 250 to 300 g. All rats were housed in individual cages under controlled temperature (22±1°C) and relative humidity under standard laboratory conditions with an artificial 12-hour light/dark cycle. All rats were allowed free access to food and water in polycarbonate units. Rats were observed for 7 days in the animal care laboratory to exclude any possibility of underlying diseases.

In sample size calculation with a power of 80% (α=0.05 and ß=0.20), we determined that 10 animals would be required in each group.

The corticosteroid-induced osteonecrosis model described by Yamamoto et al.[10] was performed. Rats were injected once with 20 mg/kg of methylprednisolone acetate (MPSL) into the right gluteus medius muscles to induce osteonecrosis 7 days after the start of feeding and allocation.

Animals were divided into two equal groups. The 10 rats in the control group (Group 1) received sterile 0.9% saline solution and soybean oil intraperitoneally at a dosage of 10 mg/kg once in five days for 4 weeks. In the CoQ_{10} group (Group 2), 10 rats received CoQ_{10} (Sigma Chemical Co., St. Louis, MO, USA) dissolved in soybean oil intraperitoneally at a dosage of 10 mg/kg once in five days for 4 weeks.[11-13]

Blood samples were obtained from the tail vein before (Week 0) and 4 weeks after (Week 4) the administration of MPLS. Samples were centrifuged and stored at -80°C until analyzed and serum malondialdehyde (MDA) and glutathione (GSH) levels were measured.

The plasma level of MDA was measured spectrophotometrically using a UV-Visible spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). The principle of the method was based on spectrophotometric measurement of the color occurring at 532 nm during the reaction to thiobarbituric acid.[14]

The plasma level of GSH was also measured spectrophotometrically on the UV-Visible spectrophotometer. The principle of the method was based on the spectrophotometric measurement of the color occurring at 412 nm during the reaction to 5,5'-dithiobis-(2-nitrobenzoic acid).[15]

Four weeks after the administration of MPSL, all rats were anesthetized with an excessive dose of sodium pentobarbital. Animals were sacrificed and the right femurs were harvested. Femoral heads were fixed in 10% buffered formalin and bone samples were decalcified with 10% formic acid. The specimens were embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin (HE).

Immunohistochemistry using the Caspase 3 (CPP32) antibody (Thermo Scientific, 1:100, rabbit) was performed on deparaffinized and rehydrated tissue sections to determine the apoptosis of the osteocytes. The number of apoptotic cells in the x200 field (Caspase 3 reaction-positive) was determined using the Nikon NIS-Elements 3.1 view analysis program.

Diagnosis of osteonecrosis was made on the basis of the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by surrounding bone marrow cell (BMC) necrosis. Only BMC necrosis in both hematopoietic and fat cells with no bone trabecula included was assessed as corticosteroid-induced osteonecrosis. Lesions composed of only a few empty lacunae within the normal bone trabecula and/or fat-cell necrosis alone were excluded from the diagnosis of corticosteroid-induced osteonecrosis.[10]

All samples were evaluated by the same pathologist without prior knowledge of which rats had been treated.

Data were analyzed using the SPSS for Windows v.19.0 (SPSS Inc., Chicago, IL, USA). Normality of continuous data was determined by the Kolmogorov-Smirnov test. Data were expressed as mean±standard deviation (SD). The Student's t-test and Mann-Whitney U test were used for differences among groups. Osteonecrosis was analyzed using the chi-square Fisher's exact probability test. Longitudinal numerical data and hematological data, (Week 0 and Week 4) were analyzed with the Wilcoxon signed-rank test. The difference between the results of hematological data was analyzed using Student's t-test. P values of <0.05 were considered statistically significant for all comparisons.

Results

Twenty Sprague-Dawley male rats were included in the experimental study. Body weight loss during the experimental period did not differ between the two groups.

Glutathione and MDA levels were similar for the

Table 1. Effects of coenzyme Q_{10} on steroid-induced osteonecrosis.

	Group 1 (Model of osteonecrosis)	Group 2 (Coenzeyme Q_{10})
Glutathione μM (Week 0)	3.2±0.2	3.3±0.3
Glutathione μM (Week 4)	1.8±0.5	2.7±0.5
Malondialdehyde nmol/ml (Week 0)	2.9±0.2	2.9±0.1
Malondialdehyde nmol/ml (Week 4)	4.2±0.9	2.9±0.5
Osteocyte count (mean)	7.90	13.10
Caspase-3 apoptosis count (mean)	14.40	6.60
Osteonecrosis (%)	70	20

control and CoQ_{10} groups before the start of the study (3.2±0.2 μM and 3.3±0.3 μM for GSH, p=0.325; 2.9±0.2 nmol/ml and 2.9± 0.1nmol/ml for MDA, p=0.791, respectively). GSH levels decreased to 1.8±0.5 in Group 1 (p=0.005) and 2.7±0.5 in Group 2 (p=0.028) at the 4th week (Table 1, Fig. 1). The decrease was significantly lower in Group 2 than to Group 1 (p=0.029). MDA levels increased to 4.2±0.9 in the control group (p=0.013) but did not change in the CoQ_{10} group (p=0.959). The increase was significantly higher in the control group than the CoQ_{10} group (p=0.009) (Table 1, Fig. 1).

Osteonecrosis was observed histologically. Seven rats (70%) in Group 1 and 2 rats (20%) in Group 2 developed osteonecrosis (Fig. 2a and b). The incidence of osteonecrosis in the CoQ_{10} group was significantly lower than that in the control group (p=0.021). Osteocyte count was less in the control group but this difference was not statistically significant (p=0.05) (Table 1).

Immunohistochemistry using the Caspase 3 (CPP32) antibody to determine the apoptosis revealed that apoptosis was less significant in the CoQ_{10} group (14.40 and 6.60 for Group 1 and 2, respectively) (p=0.002) (Table 1, Fig. 2c and d).

Discussion

Osteonecrosis of the femoral head is a serious problem that may cause severe disability and morbidity. Although severe, there is no effective medication to prevent or treat the disease. Previous studies have shown that the ratio of osteonecrosis peaks at four weeks after steroid injection in rats and oxidative stress suppresses the proliferation of osteoblasts and induces apoptosis.[7,16] Apoptosis has also been proffered as one of the mechanisms underlying steroid-induced osteonecrosis.[17] CoQ_{10} reacts with oxygen radicals, preventing direct damage of biomolecules and preventing lipid peroxidation.[18] Therefore, it has been studied in terms of cardiac, neurological, immunological and neurological diseases and shown to be protective against different forms of tissue ischemia/reperfusion damage and spinal cord trauma.[8,19-21]

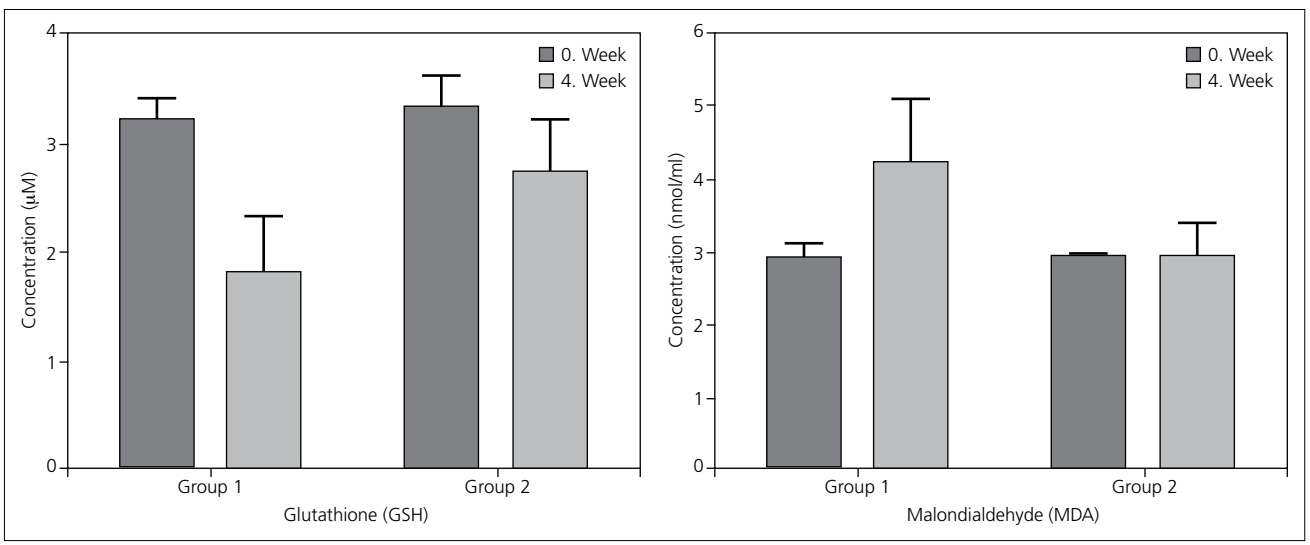

Fig. 1. Levels of GSH and MDA in the control and CoQ_{10} groups at the start of the study (0. Week) and 4th week.

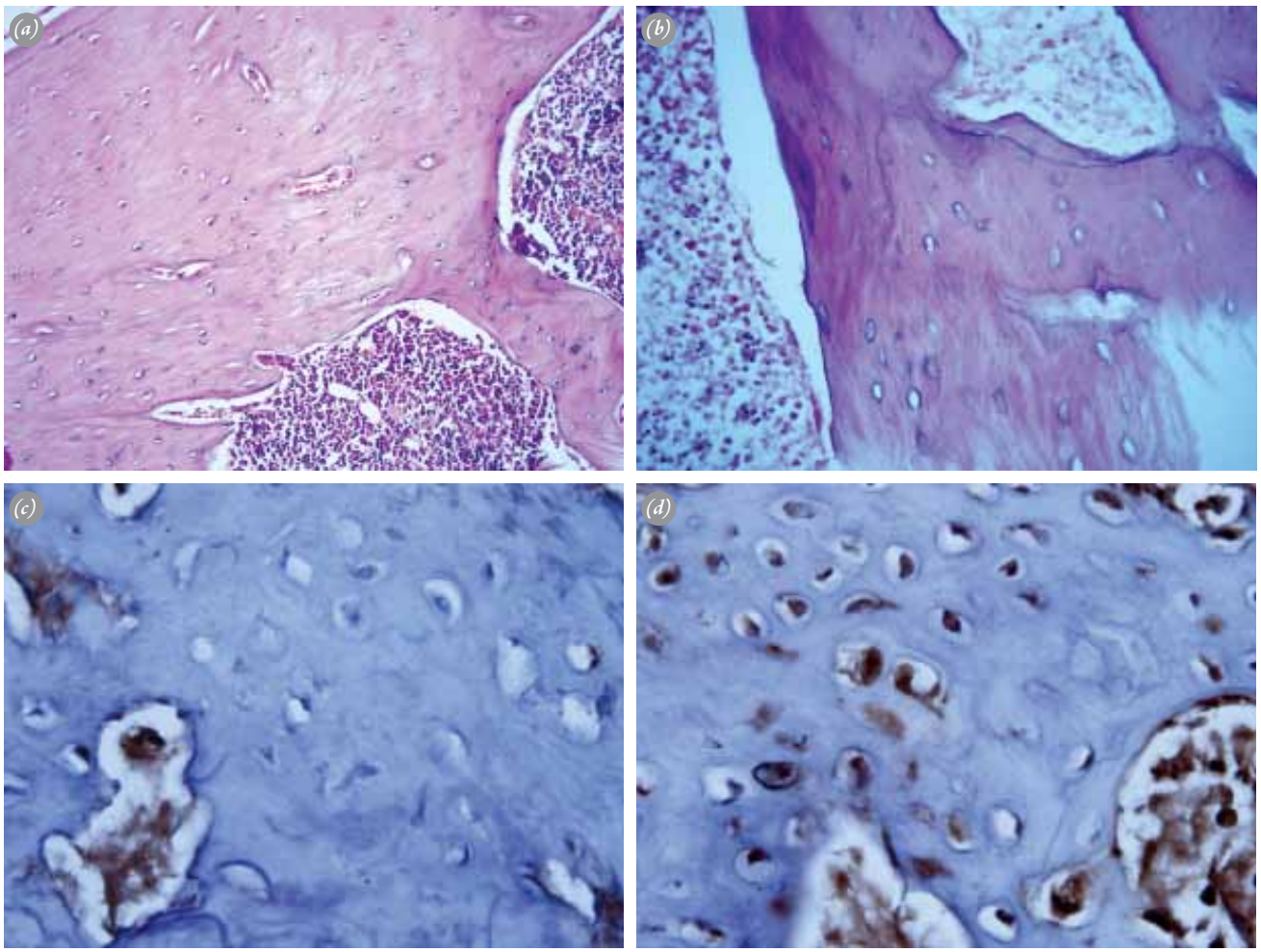

Fig. 2. **(a)** Normal osteocytes in lacunas of lamellar bone and bone marrow cells. (HE, x200). **(b)** Empty lacunas showing the osteonecrosis and coagulation necrosis of bone marrow cells (HE, x400). **(c)** Anti-Caspase 3 (-) non-apoptotic osteocytes (IHC, x1000). **(d)** Anti-Caspase 3 (+) apoptotic osteocytes (IHC, x1000). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Malondialdehyde is a product of lipid peroxidation and GSH is one of the most important antioxidants in the organism. Decreased levels of GSH and increased levels of MDA together are a significant indicator of oxidative damage.[22] Therefore, we evaluated MDA and GSH levels to evaluate the oxidative stress and found that the decrease in GSH levels and increase in MDA levels in the CoQ_{10}-treated group were less significant. This supports previous studies on antioxidants which reported that oxidative stress is inhibited by CoQ_{10}.[7,14,23] CoQ_{10} has been reported to have a protective effect against ischemic injury in experimental models.[19,21] Erol et al.[19] demonstrated that MDA levels were significantly lower in groups receiving CoQ_{10} in a testicular ischemia/reperfusion injury model. They inferred that this could be traceless to CoQ_{10} that had an antioxidant effect by lowering lipid peroxidation. Ostrowski demonstrated the neuroprotective effect of CoQ_{10} as a potent antioxidant in a cerebral ischemia model.[24] Additionally, Yenilmez et al. demonstrated that CoQ_{10} treatment appeared to ameliorate the ochratoxin A (OTA)-induced oxidative injuries in rat kidneys.[23] These findings suggest that while CoQ_{10} has a potent protective effect against oxidative damage, the protective effect of CoQ_{10} on steroid-induced osteonecrosis is still unknown.

Several animal and human studies have suggested that pharmacological approaches, including anti-coagulant agents, anti-lipid agents and antioxidant agents, may prevent steroid-induced osteonecrosis.[2-4] As we believe that the oxidation process is the major etiology of the ONFH, the potent antioxidant CoQ_{10} can be used to prevent the development of steroid-induced osteonecrosis.

In the present study, the incidence of ONFH in CoQ_{10}-treated rats was significantly lower than in non-CoQ_{10}-treated rats. Although CoQ_{10} was administrated

to prevent of ONFH, osteonecrosis still evolved in 20% of rats as osteonecrosis development is due to not only the oxidation process but also multifactorial effects.[5,7]

In our knowledge, no other experimental studies on the effect of CoQ_{10} administration on prevention of ONFH have been reported. We believe that this agent is safe to be used and could easily be tested in clinical trials to confirm its effects on prevention of osteonecrosis in humans. The major limitation of this study was the short duration of the steroid treatment in order to confirm a diagnosis of osteonecrosis. Previous studies suggest that histopathologic changes occur 2 to 20 weeks after steroid administration.[7,10,25,26] In this study, we examined the histological changes at 4 weeks, before any collapse. We presume our results, therefore, represent the early changes of osteonecrosis.

In conclusion, CoQ_{10} may be a useful agent preventing steroid-induced ONFH. Inhibited oxidative stress is a possible mechanism for this effect. The definite mechanism still requires further *in vivo* and *in vitro* studies.

Conflicts of Interest: No conflicts declared.

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