Alpha-lipoic acid could be a promising treatment in steroid-induced osteonecrosis

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Abstract. – OBJECTIVE: Glucocorticoid-induced osteonecrosis is a serious debilitating health problem. In the present study, we investigated the effects of alpha-lipoic acid on glucocorticoid-induced osteonecrosis in rats.

MATERIALS AND METHODS: A total of 40 male Wistar albino rats were equally assigned to 4 groups as control, methylprednisolone acetate (MPA), alpha-lipoic acid (ALA), and methylprednisolone acetate with alpha-lipoic acid (MPA+ALA). The animals in MPA group subcutaneously received 15 mg/kg/week for 2 weeks, whereas 100 mg/kg/day alpha-lipoic acid was intraperitoneal administered for 4 weeks to ALA group. The MPA+ALA group was subjected to both treatments in same doses. Osteonecrosis was confirmed and graded histologically. The serum concentrations of glucose, total cholesterol, low- and high-density lipoprotein, triglyceride, as well as the total oxidant and antioxidant status, oxidative stress index, prothrombin time and activated partial thromboplastin time were evaluated. Also, lipid peroxidation and DNA damage were immunohistochemically assessed in the bone.

RESULTS: Osteonecrotic lesions were narrower in the MPA+ALA group than in the MPA group ($p<0.05$). As compared to the controls, the biochemical parameters in MPA and MPA+ALA groups were significantly increased ($p<0.001$). The oxidative stress index was significantly higher in the groups with MPA than the controls ($p=0.002$), but the animals treated with ALA alongside MPA displayed lesser scores than the ones injected with solely MPA ($p=0.03$). The administration of MPA elevated lipid peroxidation and DNA damage, which were successfully alleviated by ALA.

CONCLUSIONS: Alpha-lipoic acid may be suggested to be a protective supplement in glucocorticoid-induced osteonecrosis in rats. The antioxidant capacity of alpha-lipoic acid may involve its beneficial effects.

Key Words:
   Osteonecrosis, Avascular necrosis, Methylprednisolone acetate, Alpha-lipoic acid, Oxidative stress, Rat.

Introduction

Patients suffering from a variety of rheumatological, immunological, inflammatory, and neoplastic disorders, including rheumatoid arthritis, systemic lupus erythematosus, asthma, inflammatory bowel diseases, and acute lymphoblastic leukemia, benefit from systemic glucocorticoids. Glucocorticoids act through the transcriptional activation (transactivation) and repression (transrepression), in which they positively and negatively modulate the transcription of glucocorticoid-responsive genes, respectively[1], which are also responsible for the side effects such as infections, skin changes, gastrointestinal ulceration, and mood disorders, as well as osteoporosis and osteonecrosis[2,3]. The glucocorticoid medication constitutes the leading non-traumatic cause of osteonecrosis, which may emerge even if the treatment is short-term[4]. In the United States, about 50,000 people annually undergo total joint replacement owing to steroid-induced osteonecrosis[5]. This malady significantly impairs the life quality, and creates an economic burden, since the mean age of the patients is 33 years[5].

Several events engage in the pathophysiology of steroid-induced osteonecrosis[6]. Summarily, glucocorticoids decrease the production of the bone forming and resorbing cells (i.e., osteoblasts and osteoclasts), induce the apoptosis of osteocytes, and disrupt the bone vasculature[7]. The increment of marrow adipocytes in this type of osteonecrosis leads to fat emboli, vascular occlusion, and hypercoagulability, which altogether diminish the osseous blood supply. Furthermore, glucocorticoids are known to provoke oxidative stress[3,8], and steroid-induced osteonecrosis is not exempted from its destructive consequences[9,10].

Alpha-lipoic acid is an amphipathic organosulfur compound which possesses strong antioxidant

properties and is also the coenzyme of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase[11,12]. Alpha-lipoic acid is shown to bear antidiabetic, antitumorigenic, cardioprotective, antiarrhythmic, hepatoprotective, renoprotective, and neuroprotective properties[2,13-15].

In the present study, we aimed at investigating histopathological and biochemical effects, including those of alpha-lipoic acid on the lipid profile, oxidative status, and hemostasis in an animal model of glucocorticoid-induced osteonecrosis.

Materials and Methods

Experimental Design

A total of 40 adult male Wistar albino rats, weighing 340±34 g, were assigned to 4 groups as control (n=10), methylprednisolone acetate (MPA; n=10), alpha-lipoic acid (ALA; n=10), and methylprednisolone acetate with alpha-lipoic acid (MPA+ALA; n=10). The animals in MPA group subcutaneously received 15 mg/kg/twice a week for 2 weeks, beginning from the age of 15 weeks, whereas 100 mg/kg/day alpha-lipoic acid was intraperitoneal administered for 4 weeks, beginning from the age of 13 weeks to ALA group. The MPA+ALA group was subjected to both treatments in same doses and at same times, and so, the animals were pre-treated with ALA in this group. The MPA dosing to generate osteonecrosis was determined by a previous pilot study. The animals were housed in climate-controlled rooms (22±2°C temperature, 55±10% relative humidity) under a 12:12-h light/dark cycle. Tap water and rat chow were provided *ad libitum*. MPA and ALA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Following the last treatment, animals were sacrificed under deep anesthesia acquired by a combination of ketamine and xylazine (respectively, 80 and 12 mg/kg, i.p.), and cardiac blood and femur samples were obtained.

Biochemical and Hematological Analyses

The serum concentrations of glucose, total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride (TG) were measured in an auto analyzer. The total oxidant status (TOS) and total antioxidant status[11] were evaluated from serum as defined elsewhere[16,17]. The oxidative stress index (OSI) was calculated by the ratio percentage of the total oxidant status to total antioxidant status[16,17].

Histological and Immunohistochemical Examination

The proximal parts of bilateral femur were fixed in 10% neutral phosphate buffered formalin for 24 hours, decalcified using EDTA, and embedded in paraffin. The paraffin blocks were sectioned at a thickness of 4 μm and stained with hematoxylin-eosin for examination with the light microscopy. Histopathological changes were graded as defined by Nozaki et al[18] which take the fatty degeneration, myelocyte necrosis, osteocyte necrosis, and appositional bone formation into account. Lipid peroxidation and DNA damage in the bone tissue were investigated by immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), respectively. In brief, deparaffinized sections were blocked with normal rabbit serum (diluted 1:75; Dako, Kyoto, Japan) to prevent non-specific binding. Following the incubation overnight with primary antibody for 8-OHdG or 4-HNE (Abcam, Cambridge, UK) at 4°C, the sections were incubated with secondary anti-mouse/rabbit biotinylated antibody (diluted 1:300; Dako, Kyoto, Japan), and subsequently with horseradish peroxidase-labeled streptavidin solution (diluted 1:100; Abcam, Cambridge, UK). Peroxidase activity was visualized with 3,3'-Diaminobenzidine (DAB), and counterstaining was performed with hematoxylin. The examiner was blind to the experimental groups during both histological and immunohistochemical assessments. The sections were scored with three-point method: (1) unstained, (2) somewhat stained, and (3) diffusely stained.

Statistical Analysis

The data were analyzed by one-way ANOVA followed by post-hoc Bonferroni multiple comparison test and expressed as mean ± standard error of the mean. The analyses were performed by using SPSS v.18 (IBM Corp., Chicago, IL, USA). The statistical significance threshold was set at $p < 0.05$.

Results

Lipid Profile and Blood Glucose Concentration

As shown in Table I, the administration of MPA significantly increased TC, TG, LDL, and HDL (respectively $p = 0.0001$, $p = 0.003$, $p = 0.0001$, and $p = 0.0001$). The administration of ALA alongside MPA did not change TG and HDL ($p > 0.05$),

Table I. Laboratory data and blood coagulation tests (Mean±SE).

	Control (n=10)	MPA (n=10)	ALA (n=10)	MPA+ALA (n=10)
Glucose (mg/dl)	202.0±5.5	399.8±16.5[a***]	108.2±19.8[a,b**]	303.3±15.7 [a,b***]
T. Cholesterol (mg/dl)	65.8±2.1	198.1±5.2[a***]	59.1±4.2[b***]	162.1±9.9[a***,b**]
Triglycerides (mg/dl)	51.1±5.6	104.3±22.2[a*]	47.5±8.6[b**]	81.8±9.7[a*]
LDL (mg/dl)	35.3±1.2	112.0±5.1 [a***]	29.6±2.0[b***]	91.1±7.3[a***,b*]
HDL (mg/dl)	23.7±1.0	47.2±2.5[a***]	57.8±2.4[a***,b*]	53.5±2.7[a***]
LDL/HDL ratio	1.50±0.08	2.40±0.12[a***]	0.52±0.04[a,b***]	1.73±0.16[a***,b**]
PT	13.79±0.2	15.9±0.6[a*]	14.8±0.6	14.0±0.2[b*]
INR	1.06±0.04	1.23±0.06[a*]	1.09±0.04	1.09±0.01[b*]
aPTT	37.5±2.0	43.5±4.3	30.8±4.5[b*]	30.5±0.8[b*]

The mean difference is significant at the level of 0.05 ($p<0.05$). ANOVA Test and Student's t-test used. MPA, Methylprednisolone acetate; ALA, Alpha Lipoic acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PT, prothrombin time; aPTT, activated partial thromboplastin time; INR, International normalized ratio.
[a]: *vs.* Control, [b]: *vs.* MPA
*: $p<0.05$, **: $p<0.001$, ***: $p<0.0001$

but TC and LDL (respectively $p = 0.006$ and $p = 0.001$), although there was a significance between the MPA+ALA group and controls for all lipid profile parameters ($p < 0.05$). The LDL/HDL ratio was significantly higher in the MPA group ($p = 0.0001$) and decreased with the treatment of ALA ($p = 0.006$). The animals with only ALA had the highest concentrations of HDL, which was statistically significant in comparison to the MPA and control groups (respectively $p = 0.01$ and $p = 0.0001$). There was no difference for TC, TG, and LDL between the ALA group and controls ($p > 0.05$).

The blood glucose levels were increased with MPA ($p = 0.0001$), and ALA achieved to decrease blood glucose ($p = 0.0007$); however, it did not reach to the extent of that was seen in controls ($p = 0.0001$). There was significance between the ALA group and controls ($p = 0.0005$) (Table I).

Hemostatic Parameters

The animals with solely MPA showed an increase of PT/INR ($p = 0.03$), but not of aPTT ($p > 0.05$). The administration of ALA alongside MPA changed PT/INR and aPTT ($p = 0.04$ and $p = 0.003$, respectively), There was no statistical significance for the hemostatic parameters between the MPA+ALA group and controls ($p > 0.05$) (Table I, Figure 1).

Oxidative Status

As shown in Table II, there was no significance for the TAS between the experimental groups ($p > 0.05$). The TOS levels were higher in MPA admin-

istered groups (MPA and MPA+ALA) than the controls (respectively, $p = 0.003$ and $p = 0.005$), although the animals with MPA+ALA showed significantly lower values than the MPA group ($p = 0.003$). Since the OSI a ratio percentage of the TOS and TAS, glucocorticoid-injected animals displayed an increment of OSI as compared to the controls ($p < 0.05$), and in comparison, to the MPA group, the administration of ALA alongside MPA decreased the OSI ($p = 0.03$), although it was still higher than the controls ($p = 0.04$) (Figure 2).

As demonstrated in Table III and Figure 3, immunohistochemical staining of the femur sections for 8-OHdG and 4-HNE was more frequent in the MPA group (respectively, $p = 0.005$ and $p = 0.002$). On the other side, 8-OHdG- and 4-HNE-positive cells were decreased in the animals with MPA+ALA as compared to the MPA group (respectively, $p = 0.02$ and $p = 0.02$). There was no significance between the ALA group and controls ($p > 0.05$).

Histopathological Examination

As shown in Table III and Figure 4, there was no histopathological finding in the controls. The occurrence of fatty degeneration was higher in the MPA group than in the others ($p < 0.05$). Myeloid necrosis and osteocyte necrosis were not observed in the ALA and MPA+ALA groups, but only in the MPA group. None of the animals displayed new bone formation. According to previously mentioned classifications, the incidence of osteonecrosis was 50% in the MPA group, whereas no sign of it was seen in the MPA+ALA group.
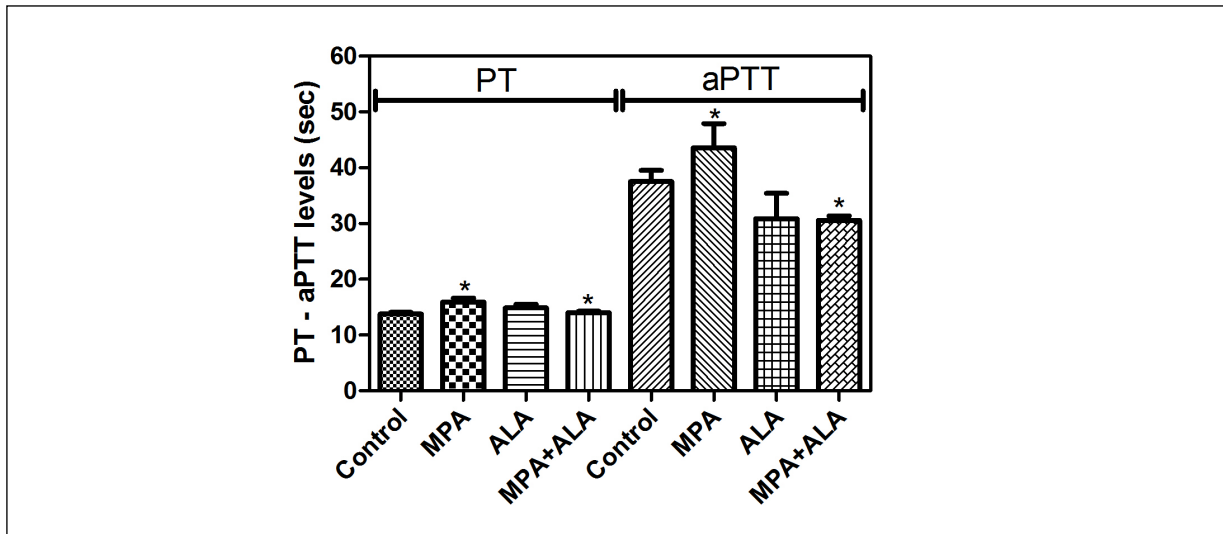
Figure 1. Comparison of PT and aPTT levels in the groups. PT, prothrombin time; aPTT, activated partial thromboplastin time; MPA: methylprednisolone acetate; ALA: alpha lipoic acid; TAS: total antioxidant status; TOS: total oxidative status; OSI: oxidative stress index.

Discussion

Glucocorticoid-induced osteonecrosis is a serious debilitating health problem. Reduced blood supply is conventionally attributed to the pathophysiology of the disease as the preeminent factor[19], even though there is no definitive explanation yet. Hypercoagulability, which promotes the formation of thrombi, and fat emboli, which is a result of disturbed lipid metabolism, are proposed to occlude osseous blood flow[19].

Some of the Cushing syndrome patients carrying von Willebrand Factor (vWF) haplotype 1 display increased vWF which seems to be linked with thrombotic events[20,21]. However, in a systemic review by van Zaane et al[22], hypercoagulability-related complications are suggested to probably be a result of the overlap of the exogenous glucocorticoid administration and underlying disease rather than a direct effect of the treatment, without ignoring diminishment of fibrinolytic activity by glucocorticoids. Although common intuition is a hypercoagulable state with glucocorticoids, Pandit and Spillert[23] have reported that high-dose methylprednisolone treatment resulted in hypocoagulability, in concordance with our results. Importantly, it should be noted that healthy laboratory animals were recruited in our study and hence, we do not neglect the possibility that glucocorticoids may favor hypercoagulability in inflammatory or neoplastic pathologies. Also, in the medical practice, the treatment is usually long-term, and our model is not appropriate for a presumption of long-term effects of glucocorticoids on hemostatic parameters.

Table II. Levels of oxidative status in groups (Mean±SE).

	Control (n=10)	MPA (n=10)	ALA (n=10)	MPA+ALA (n=10)	*p*-value
TAS (mmol/L)	1.40±0.8	1.48±0.3	1.13±0.0	1.28±0.0	
TOS (μmol/L)	57.46±12.3	214.9±27.9*	42.14±4.5	117.5±10.6**	*:=0.0003 **:=0.003
OSI (=TOS/TAS)	48.2±13.2	148.0±23.6*	33.8±2.7	89.2±9.0**	*:=0.002 **:=0.03

The mean difference is significant at the level of 0.05 (*p*<0.05). ANOVA Test and Student's *t*-test used. MPA: methylprednisolone acetate; ALA: alpha lipoic acid; TAS: total antioxidant status; TOS: total oxidative status; OSI: oxidative stress index. *: MPA *vs.* Control; **: MPA *vs.* MPA+ALA.
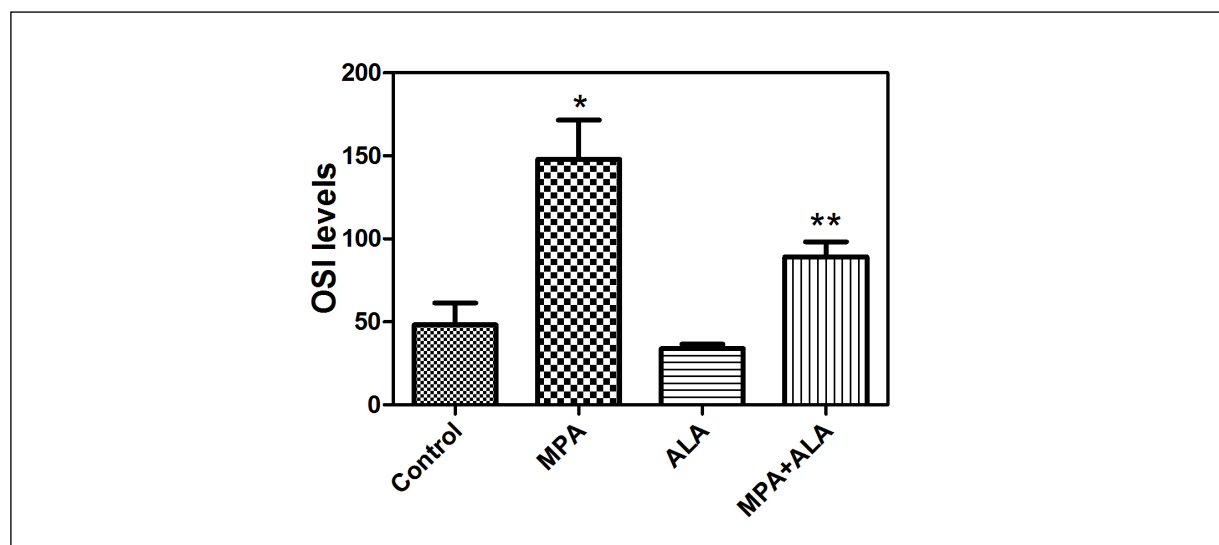
Figure 2. Comparison of oxidative stress index in the groups. MPA: methylprednisolone acetate; ALA: alpha lipoic acid; TAS: total antioxidant status; TOS: total oxidative status; OSI: oxidative stress index.

Hyperlipidemia is a well-defined adverse effect of the glucocorticoid treatment which involves in the diminishment of the blood supply in glucocorticoid-induced osteonecrosis[24]. It contributes to thrombosis by increasing the viscosity of blood[25]. Based on this fact, several authors[26,27] have reported that lipid-lowering agents improve glucocorticoid-induced osteonecrosis. Not surprisingly, we found that the administration of methylprednisolone acetate leads to hyperlipidemia, as previous reports have confirmed[26,27].

Oxidative stress can simply be described as the disruption of the redox balance favoring pro-oxidation. Glucocorticoids induce oxidative stress by both escalating pro-oxidants and weakening the antioxidant defense[28]. Oxidative stress is suggested to play a role in the apoptotic events in glucocorticoid-induced osteonecrosis[29]. Ichiseki et al[30] have reported that following the administration of methylprednisolone, oxidative injury is evident before the emergence of osteonecrotic lesions. As mentioned before, hyperlipidemia is one of the core difficulties in glucocorticoid-induced osteonecrosis, and oxidative stress may be aggravated by hyperlipidemia, which exacerbates the tissue damage[29]. In the present study, we found that the treatment of methylprednisolone acetate exacerbated the total antioxidant status

Table III. The scores of histopathology and immunohistochemistry.

	Control (n=10)	MPA (n=10)	ALA (n=10)	MPA+ALA (n=10)
Fatty degeneration	0.00±0.00	1.11±0.1	0.16±0.1	0.33±0.2[b*]
Myeloid necrosis	0.00±0.00	0.37±0.48	0.00±0.00	0.00±0.00
Osteocyte necrosis	0.00±0.00	0.44±0.17	0.00±0.00	0.00±0.00
New bone formation	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Osteonecrosis	0.00±0.00	2.00±0.26	0.00±0.00	1.22±0.22[b*]
4-HNE	0.85±0.14	2.25±0.25[a*]	1.16±0.30	1.28±0.28[b*]
8-OHdG	1.14±0.26	2.37±018[a*]	1.33±0.33	1.57±0.20[b*]

MPA: methylprednisolone acetate; ALA: alpha lipoic acid; 4 –HNE: 4-hydroxy-2-nonenal; 8-OHdG: anti-8-hydroxy-2′-deoxyguanosine.
[a] MPA *vs.* control, [b] MPA+ALA *vs.* MPA.
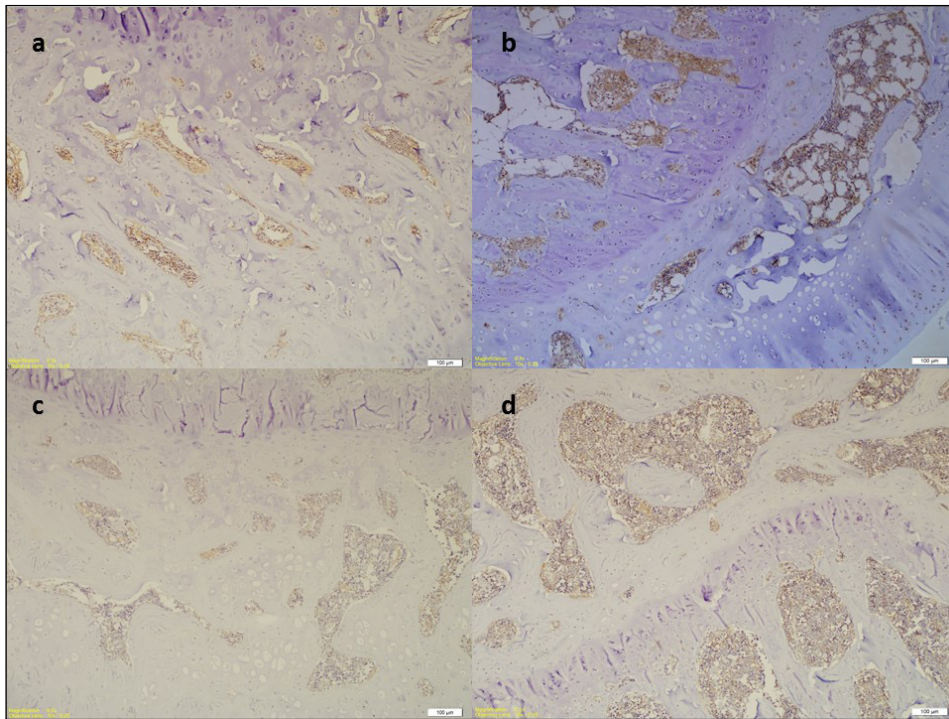*: $p < 0.05$, **: $p < 0.001$.

Figure 3. Immunohistochemical staining of the femur sections in groups (x100): **a**, Control; **b**, methylprednisolone acetate (MPA); **c**, alpha lipoic acid (ALA); **d**, MPA + ALA.

and oxidative stress index, while decreasing total antioxidant status, in the serum as well as 8-OHdG (DNA damage marker) and 4-HNE (lipid peroxidation marker) in the bone tissue. Our findings are in accordance with previous reports that point out oxidative stress in glucocorticoid-induced osteonecrosis[9,10,29,30].

The data from experimental studies indicate that even a single injection of glucocorticoids can generate osteonecrosis[19,31], and the necrotic lesions are obvious 2 weeks after the injection[24]. Thus, we implemented a model of biweekly administration of methylprednisolone acetate in a course of 2 weeks and observed the findings of osteonecrosis of the femur head. The alpha-lipoic acid treatment was begun 2 weeks earlier than the glucocorticoid injections to reveal any protective effect. We demonstrated that alpha-lipoic acid successfully improves glucocorticoid-induced osteonecrosis in these experimental settings. Our result was similar to that reported in a study by Lu and Li[32], in which they have used a lower dose of alpha-lipoic acid (36 mg/kg/day for 4 weeks) in rabbits. In that study, the incidence of osteonecrosis was reported to be 20.8% in steroid-injected and 73.1% in alpha-lipoic acid supplemented animals, whereas

we found an incidence of 50% in the steroid-injected group and no osteonecrosis in the latter one. This difference may be a result of higher alpha-lipoic acid dose used in our study. Nonetheless, both studies point out an improvement with alpha-lipoic acid in steroid-induced osteonecrosis. The benefits of alpha-lipoic acid in our study may originate from its effects on the lipid profile and/or redox balance. It decreased the concentrations of total cholesterol and LDL, whereas did not alter HDL and triglycerides. The LDL/HDL ratio, which reflects the extent of lipid transport to peripheral tissues, is predictive for the intensity of osteonecrosis, and the animals with alpha-lipoic acid displayed lower LDH/HDL ratio in the present study. To our results, alpha-lipoic acid significantly mitigates oxidative stress in the serum, which was estimated by the total oxidant status and total antioxidant status as well as by oxidative stress index. This was in accordance with the results of abovementioned study by Lu and Li[10,32,1,2], in which they have evaluated oxidative status by measuring glutathione and malondialdehyde levels in the plasma. In our study, we also immunohistochemically investigated oxidative injury in the bone tissue and noted that alpha-li-
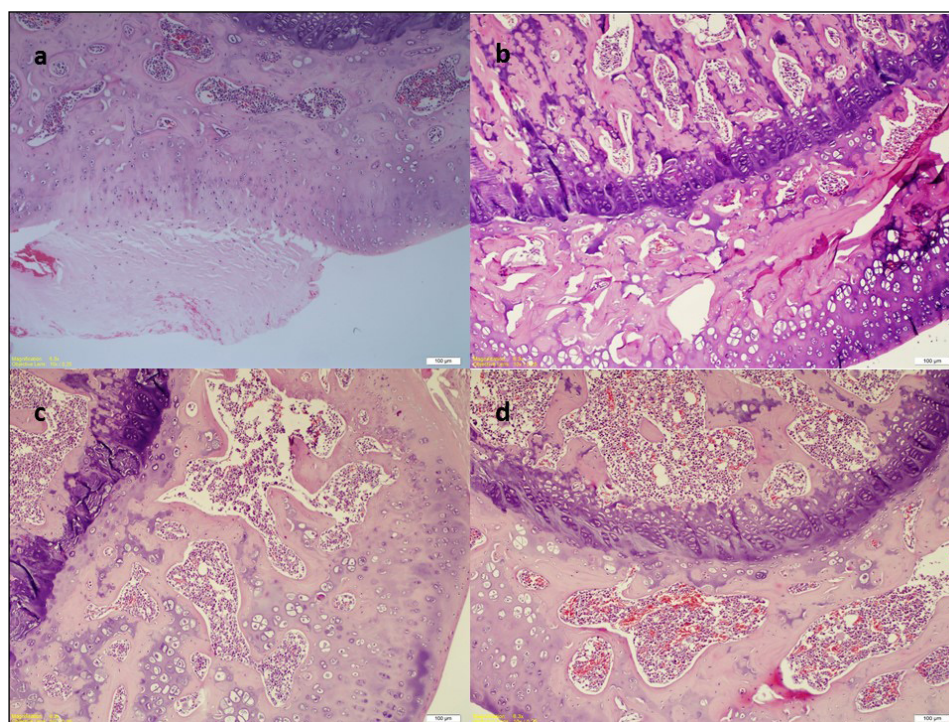
Figure 4. Hematoxylin-eosin staining of the femur sections in groups (x100). **a**, Control; **b**, methylprednisolone acetate (MPA); **c**, alpha lipoic acid (ALA); **d**, MPA + ALA.

poic acid relieves DNA damage and lipid peroxidation, which were respectively assessed by 8-OHdG and 4-HNE, in glucocorticoid-induced osteonecrosis.

Limitations

A major limitation of this study is the focus on oxidative stress and lipid disruption, which are not the only events that cause steroid-induced osteonecrosis. Further researches are required on the effects of Alpha-lipoic Acid on this complex pathology.

Conclusions

To date, there is no effective in-use medical treatment for glucocorticoid-induced osteonecrosis, although several molecules, including anti-coagulants, anti-hyperlipidemics, and anti-oxidants, are suggested to be useful. Depending on our results, we concluded that alpha-lipoic acid may alleviate glucocorticoid-induced osteonecrosis. Nevertheless, the present pre-clinical study did not aim at revealing an ideal dose of alpha-lipoic acid to use against steroid-induced osteonecrosis in clinical practice. However,

studies with large clinic studies are needed on this subject.

Conflict of Interest

The authors declare that they have no potential conflicts of interest.

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Ethics Approval

The experimental procedures were approved by the Local Ethics and Animal Care Committee of Mustafa Kemal University and performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

Authors' Contributions

Concept – R.D., I.K., M.Y., H.O.A., M.U.; Design – R.D., I.K., M.Y.; Wiritng – I.K., R.D. M.Y., H.O.A., M.U.; Supervision – R.D., I.K., M.Y., H.O.A., M.U.; Data Collection and/or Processing – I.K., M.Y., H.O.A., M.U. R.D.

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