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Effects of Alpha-Lipoic Acid on TGF-β1 and Urotensin-II Levels in Glucocorticoid-Induced Osteonecrosis in Rats

Irfan Koca [1], Recep Dokuyucu [2,*], Zeynel Abidin Tas [3], Hasan Gokce [4] and Oguzhan Ozcan [5]

[1]Department of Physical Medicine and Rehabilitation, Special Osmaniye Sevgi Hospital, Osmaniye, Turkey
[2]Department of Physiology, Medical Specialty Training Center (TUSMER), Ankara, Turkey
[3]Department of Pathology, City Hospital, Adana, Turkey
[4]Department of Pathology, School of Medicine, Inonu University, Malatya, Turkey
[5]Department of Biochemistry, School of Medicine, Mustafa Kemal University, Hatay, Turkey

[*]*Corresponding author*: Department of Physiology, Medical Specialty Training Center (TUSMER), Ankara, Turkey. Email: drecepfatih@gmail.com

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Abstract

Background: Osteonecrosis (ON) is a serious health problem, which dramatically reduces the quality of life.
Objectives: In the present study on the rat model of glucocorticoids (GCs) -induced ON, we explored the influence of alpha-lipoic acid on serum levels of TGF-β1 and urotensin-II (U-II) and on histological alteration with respect to fatty degeneration and osteocyte necrosis.
Methods: A total of 32 male Wistar albino rats were equally assigned to four groups, including control, methylprednisolone acetate (MPA), alpha-lipoic acid (ALA), and MPA with ALA (MP1 + ALA). The animals in MPA group subcutaneously received 15 mg/kg/week during 2 weeks, whereas 100 mg/kg/day ALA was intraperitoneally administered to ALA group during 4 weeks. The MPA + ALA group had both treatments with the same doses. ON was confirmed and graded histologically. Lipid peroxidation and DNA damage levels were immunohistochemically assessed in rats' bones.
Results: After histopathological examinations, ALA injection attenuated oxidative stress levels through reducing both 8-OHdG-and 4-HNE-positive cells in the femoral head region ($P < 0.05$). The U-II and TGF-β1 protein levels significantly decreased after ALA treatment in MPA injected animals ($P < 0.05$, $P < 0.01$, respectively). Moreover, there was a strong correlation between U-II and TGF-β1 protein levels ($P = 0.019$, $r = 0.884$).
Conclusions: This study is novel with regard to showing the therapeutic effects of ALA on GC-induced ON in rats as well as the strong correlation between the expression levels of U-II and TGF-β1 proteins. In this regard, ALA may be a therapeutic agent in the treatment of ON patients.

Keywords: Osteonecrosis, Alpha-Lipoic Acid, Urotensin-II, TGF-β1

1. Background

Chronic inflammation is the leading cause of vascular-flow impaired diseases such as atherosclerosis, liver cirrhosis, pulmonary fibrosis, hypertension, diabetic retino- and nephropathies, and heart and renal failures (1-3). Due to their anti-inflammatory actions performed through inducing apoptosis and/or cell-cycle arrest of inflammatory cells such as T cells and eosinophils (4), glucocorticoids (GCs) are the most common therapeutic option worldwide in the treatment of inflammatory diseases. On the other hand, severe side-effects such as dyslipidemia and hypertension, infections, depressive symptoms, muscle weakness, skin irritations, obesity, diabetes mellitus, osteoporosis, and osteonecrosis (ON) (5-10) have been reported for the use of corticosteroid-based drugs in long-term.

In this regard, although there is no consensus among scientists, much evidence is observed on the association between periodic GC use and non-traumatic ON pathology regarding the dose and duration of GC treatment (10-12). The estimated incidence of steroid-induced ON is 10,000 - 20,000 per year in the United States, accounting for 2 - 4% of total joint replacement operations per year (11). Furthermore, 9 - 40% of patients treated either for a long- or short-term period using a high-dose of GCs develop non-traumatic ON (13).

U-II is a cyclic oligopeptide, which is originally isolated from nervous system of teleost fish and widely expressed in mammals (14). The first detected function of U-II was to act as a vasoconstrictive agent besides its pro-fibrotic role in the heart (15). In an in-vitro rat model, U-II was shown to

be involved in collagen synthesis in cardiac fibroblasts via ERK1/2-dependent and independent pathway using TGF-β1 stimulation (16). The symptoms of several diseases of the impaired circulation system such as atherosclerosis, hypertension, heart failure, diabetes, and renal failure have a feature in common, i.e. the elevation of U-II levels (17-21). In a pulmonary fibrosis rat model, Onat et al. (22) showed that U-II receptor antagonist treatment alleviated the fibrosis in lung and significant reduced the increased levels of TGF-β1 and U-II after pulmonary fibrosis. In line with this finding, it was shown that U-II antagonist in a rat model of cirrhosis inhibited the fibrogenesis in cirrhotic liver tissue (23). Moreover, it was claimed that U-II treatment increased collagen-I synthesis via TGF-β1 signaling pathway in rat aortic vascular smooth muscle cells (24). Accordingly, it can be concluded that U-II and TGF-β1 have pivotal functions in the progression of vascular fibrosis.

Furthermore, a study on osteoarthritic patients revealed that the U-II levels in the knee synovial fluid of the patients were significantly higher, compared to the healthy control group, suggesting that U-II may play an important role via inducing TGF-β pathway leading to synovial fibrosis (25). In the support of these findings using a diabetes rat model, it was asserted that U-II upregulation during the course of the disease led to TGF-β1-mediated renal fibrosis (15).

TGF-β isoforms (1, 2, and 3) have role in ECM production by modulating mesenchymal cell proliferation and homeostasis as well as fibrogenesis in several tissues as their primary role (26). As it was mentioned, although TGF-β1 expression is not solely depended on U-II (27), the expressions of U-II and TGF-β1 show similar pattern (not in all types) during the ON. In a clinical study on the patients bearing bisphosphonate-associated ON of the jaw (Bronj) disease and osteoradionecrosis, although the expression of TGF-β1 was revealed to decrease in bronj tissue, it increased in osteoradionecrosis tissue (28). In another articular osteoarthritis (OA) mouse model, adenoviral TGF-β overexpression caused persistent synovial fibrosis through the upregulation of lysyl hydroxylase 2 in knee and consolidated OA (29). On the other hand, although exogenous injection and adenoviral overexpression of TGF-β were involved in fibrogenesis in osteoarthritis animal models, endogenous TGF-β was reported to have a critical protective function against cartilage lose (30, 31).

Alpha-lipoic acid (ALA), selected as our target molecule to treat GC-induced ON in rats, is a mitochondrial cofactor, which can easily interact with radical and metal groups as a metal chelator (32). Accordingly, as an effective antioxidant molecule, ALA has been commonly used as a therapeutic agent in diabetes mellitus, cancer, cardiovascular disease, and liver diseases with minimum side effects (32-37). We selected ALA as our target therapeutic molecule in steroid-induced ON since it can function as a strong antioxidant and anti-inflammatory agent. It is also documented that ALA ameliorates impaired vascular endothelium (32, 38).

2. Objectives

This study aimed to analyze the therapeutic effects of ALA treatment in GC-induced osteonecrotic rats through measuring fatty acid degeneration, ON levels, oxidative status, and TGF-β1 and U-II protein levels.

3. Methods

3.1. Experimental Design

In this study, 32 adult male Wistar albino rats (weight, 340 $\pm$ 34 g) were assigned to four groups: control (n = 8), MPA (n = 8), alpha-lipoic acid (ALA; n = 8), and MPA with ALA (MPA + ALA; n = 8). MPA (Sigma-Aldrich, St. Louis, MO, USA) was injected to 15-week-old animals subcutaneously in order to create ON at a dosage of 15 mg/kg twice a week during two weeks, as previously described in a pilot study. ALA (Sigma-Aldrich) was injected to 13-week-old animals intraperitoneally at a dosage of 100 mg/kg per day during four weeks. The animals in MPA + ALA group were treated with the same dosage during the same time period, i.e. ALA treatment was performed 2 weeks prior to the MPA injection. The animals were taken care in special rooms under 22 $\pm$ 2°C with 55 $\pm$ 10% humidity in the controlled 12h-long light/dark cycle periods. Animals were fed with rat chow and tap water ad libitum. At the end of the treatment, all the animals were sacrificed with an anesthesia cocktail (80 mg/kg ketamine and 12 mg/kg xylazine i.p.). Thereafter, cardiac blood and femur samples were collected for further biochemical and histological analyses. All the experiments were in accordance with the Local Ethics and Animal Care Committee of Mustafa Kemal University (code: #2014-07/01) and the National Institute of Health Guide for Care and use of Laboratory Animals (publication no.: 86 - 23, revised 1996).

3.2. Biochemical and Hematological Analyses

The serum levels of TGF-β1 and Urotensin-II were measured using a commercial rat Enzyme-Linked Immunosorbent Assay (ELISA) kit (YH Biosearch Laboratory, China). Detectable U-II and TGF-β1 levels range from 5 to 1000 ng/mL. The sensitivity of U-II ELISA Kit is 2.23 ng/mL. In short, blood serum samples were centrifuged, and the supernatants

were loaded into 96 microwell plates. Biotinylated anti-TGF-1β and anti-urotensin-II antibodies and streptavidin-HRP conjugate were added to the samples and incubated for 60 minutes at 37°C after a short mix. After 5× washing, the samples were exposed to chromogen reagent mixture for 10 minutes at 37°C. Finally, the reaction was ended with the stop solution, and measurements were performed at 450-nm wavelength with a spectrophotometer.

3.3. Histopathological and Immunohistochemical Analysis

Following the fixation of bilateral femur with 10% formalin for 24 hours and decalcification with EDTA, the samples were embedded into paraffin blocks. Then 4 μm-thick paraffin sections were produced by a microtome, and the slices were deparaffinized in xylol and a descending series of ethanol for further histological (hematoxylin-eosin staining) and immunohistochemical (IHC) analysis. The histopathological analysis was performed as described previously by Nozaki et al. (39). Fatty degeneration and osteocyte necrosis are the main criteria which were analyzed according to Nozaki et al. (39) With using 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) IHC assays, the lipid peroxidation and DNA damage in the bone tissue were measured, respectively. To summarize, deparaffinized tissue sections were treated with rabbit serum (dilution; 1:75; Dako, Kyoto, Japan) to block non-specific binding. Then the samples were incubated at 4°C overnight using recommended dilutions of anti-8-OHdG or anti-4-HNE primary antibodies (Abcam, Cambridge, UK). Following the incubation with biotinylated secondary anti-mouse or anti-rabbit antibody (dilution; 1:300; Dako) for 1 hour at room temperature, the sections were treated with horseradish peroxidase-labeled streptavidin (dilution; 1:100; Abcam, Cambridge, UK) for another 1 hour and then exposed to 3,3'-diaminobenzidine (DAB) for chromogenic visualization of peroxidase activity. The samples were counterstained with hematoxylin and examined/analyzed with light microscope by a blind examiner, who was unaware of the experimental groups. Staining was scored using a three-point Likert scale; (1) unstained, (2) somewhat stained, and (3) diffusely stained (39).

3.4. Statistical Analyses

One-way ANOVA and post hoc Bonferroni multiple comparison tests were used to assess statistical differences between the groups. The data were demonstrated as the mean ± standard error of the mean (SEM), and the significance level was set to be 0.05. All the statistical analyses were calculated with SPSS software version 18 (Chicago, IL, USA).

4. Results

4.1. Histopathological Examination

Following the histopathological examinations and hematoxylin and eosin staining, fatty degeneration and ON levels were measured (Figure 1, Table 1). The fatty degeneration in the MPA + ALA group showed significant reduction, compared to the control and MPA-treated groups ($P < 0.05$, Table 1). Moreover, as shown in Figure 1B, the increased levels of fatty degeneration in MPA-treated tissue resulted in numerous unstained holes arising from elevated lipid steatosis and more intense dark blue hematoxylin staining. This was caused by an increased oxidation within the tissue, compared to the other groups.

There was no measurable ON pattern in the control and ALA groups. Moreover, ALA treatment in MPA-exposed tissue reduced ON levels significantly, compared to MPA group ($P < 0.05$, Table 1, Figure 1). This remarkable change was also noticed in H & E stained MPA-treated tissue, which lost tissue integrity and cellular mass due to an increase in ON (Figure 1B and D). The difference between the ALA group and the control groups was not significant. The animals experienced no new bone formation. As previously mentioned classification, the portion of osteonecrotic animals was 50 % in the MPA group while ON was detected fewer in the MPA + ALA group.

4.2. Oxidative Status

As demonstrated in Figure 2 and Table 1, IHC examination and analysis of the femur sections revealed that the number of 8-OHdG and 4-HNE positive cells after MPA treatment increased significantly, compared to the control groups ($P < 0.05$).

To support these findings, it can be obviously noticed in Figure 2B that, because of lipid steatosis, large holes together with increased number of 8-OHdG and 4-HNE positive cells were formed in MPA-treated tissue. As shown in Figure 2D, ALA treatment significantly decreased the number of 8-OHdG- and 4-HNE-positive cells in MPA + ALA group, compared to the MPA group ($P < 0.05$, Table 1). The difference between the ALA and the control groups was not significant.

4.3. TGF-β1 and U-II expression

The protein levels of TGF-β1 and U-II were measured with ELISA assay (Figure 3). It was observed that MPA treatment increased the protein levels of both TGF-β1 (337.60 ± 13.01 pg/mL) and U-II (199.5±5.0 ng/ml) significantly, compared to the control groups (229.9±17.35 pg/ml and 158.10 ± 8.20 ng/mL respectively, $P < 0.001$). Besides, TGF-β1 and

Table 1. The Scores of Histopathology and Immunohistochemistry[a]

	Control (N = 7)	MPA (N = 8)	ALA (N = 7)	MPA + ALA (N = 8)
Fatty degeneration	0.00 ± 0.00	1.44 ± 0.1	0.50 ± 0.2	0.66 ± 0.3[b, c]
Osteonecrosis	0.00 ± 0.00	2.30 ± 0.39	0.00 ± 0.00	1.52 ± 0.25[b, c]
4-HNE	1.18 ± 0.47	2.58 ± 0.12[b, c]	1.49 ± 0.20	1.59 ± 0.18[b, c]
8-OHdG	1.47 ± 0.39	2.70 ± 0.25[b, c]	1.65 ± 0.12	1.60 ± 0.32[b, c]

bbreviations: ALA: alpha lipoic acid; MPA, methylprednisolone acetate; 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, anti-8-hydroxy-2'-deoxyguanosine.
[a]Values are expressed as mean ± SD.
[b]MPA vs control; b MPA + ALA vs MPA.
[c]P < 0.05.

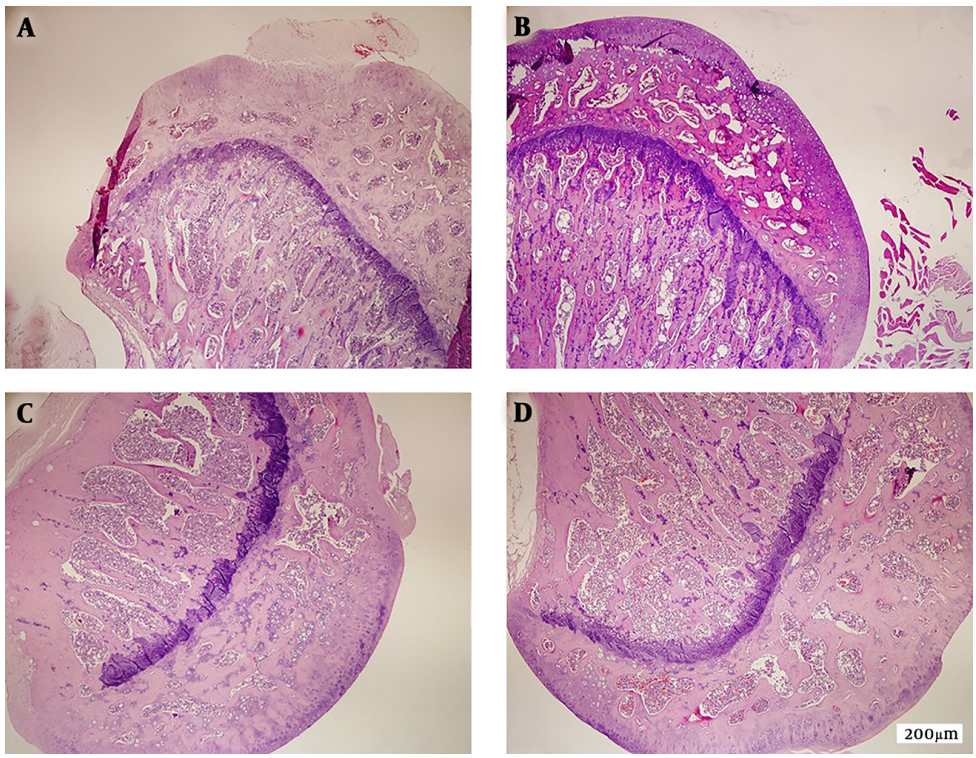

Figure 1. Hematoxylin-eosin staining of femur sections. A, Control; B, MPA; C, ALA; and D, MPA + ALA. (MPA, methylprednisolone acetate; ALA, alpha lipoic acid, 100×, scale bar: 200 μm).

U-II expressions in MPA-treated groups were also significantly higher, in comparison to the ones in ALA-treated groups (248.20 ± 11.36 pg/mL, P < 0.01 and 151.20 ± 3.45 ng/mL, P < 0.001, respectively). Moreover, ALA treatment together with MPA decreased the TGF-β1 (273.50 ± 18.38 pg/mL) and U-II (162.10 ± 5.2 ng/mL) protein levels significantly, compared to MPA-only-treated groups (P < 0.05 and P < 0.01, respectively).

There was a statistically positive correlation (P < 0.05) between TGF-β1 and U-II protein levels in all the study groups (Figure 4A, C and D, rcontrol = 0.920, rALA = 0.892, rMPA + ALA = 0.884); however, a statistically negative correlation was observed in MPA group (Figure 4B, rMedrol = -0.830) (P < 0.05).

5. Discussion

As it is well-documented, one of the reasons of non-traumatic ON is the GC use for therapeutic purposes against chronic inflammation, which is the main cause of vascular flow impaired diseases (40). Although GC-induced ON proceeds in a multifocal fashion, the most common affected region is femoral head (40). Although

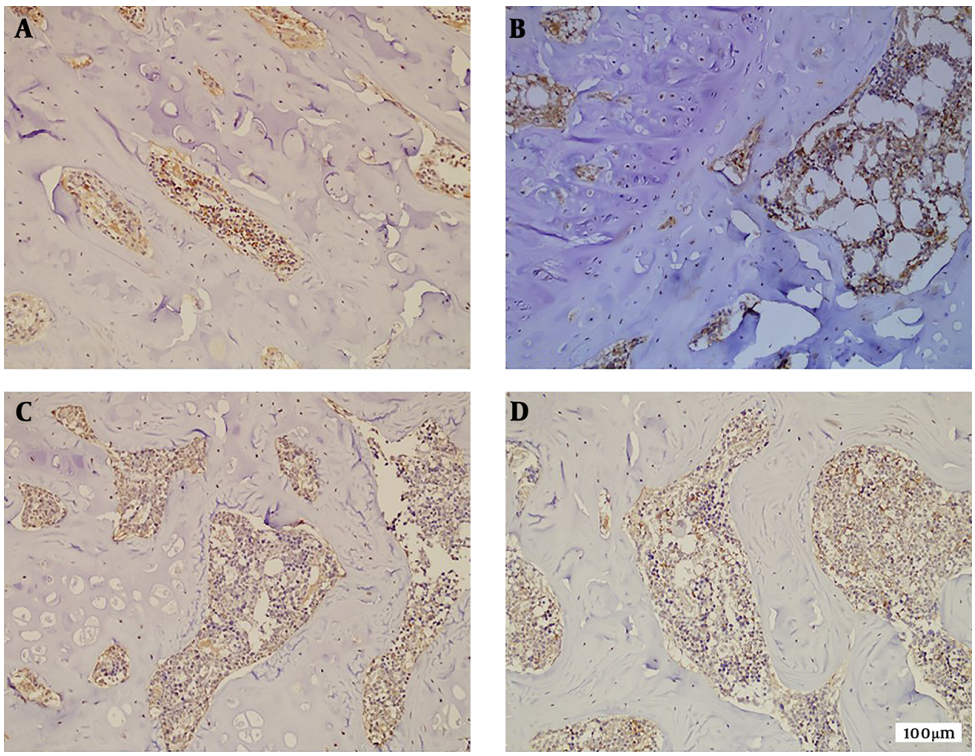

Figure 2. Immunohistochemical staining of femur sections in the study groups (4-hydroxy-2-nonenal and anti-8-hydroxy-2'-deoxyguanosine). A, Control; B, MPA; C, ALA; and D, MPA + ALA. (MPA, methylprednisolone acetate; ALA, alpha lipoic acid, 100 ×, scale bar, 100 μm).

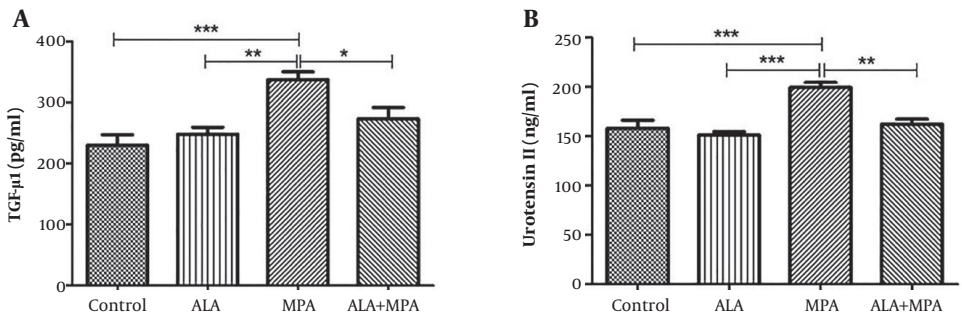

Figure 3. A, The TGF-β1; B, and U-II protein levels were measured with ELISA. The bar graphs, designated with different motifs for each group, represent the TGF-β1 (pg/mL) and U-II (ng/mL) protein levels in control, ALA, MPA and MPA + ALA groups. MPA, methylprednisolone acetate; ALA, alpha lipoic acid. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

there is no consensus among scientists regarding the direct relationship between ON pathophysiology and therapeutic GC uptake, several experimental models have established a strong correlation between GC treatment and ON progression. In a rabbit model of ON, even a one-shot-high-dose injection of GC was sufficient to cause ON in different bones of the animals (41). In this regard, in another ON model with pigs, high dose OC treatment reduced osseous blood flow in the early stage of ON (42). In another rabbit model of GC-induced ON, LDL/HDL cholesterol levels were observed to be significantly high, implying the elevated levels of marrow fat mass (43).

Previously, it was shown that MPA (methylprednisolone) treatment makes blood samples of healthy donors gain a hypocoagulable status (44). In a similar vein, in a rabbit model of GC-induced osteonecrosis, aberrant hypercoagulability was observed to develop thrombus (45). These contradictory findings are caused

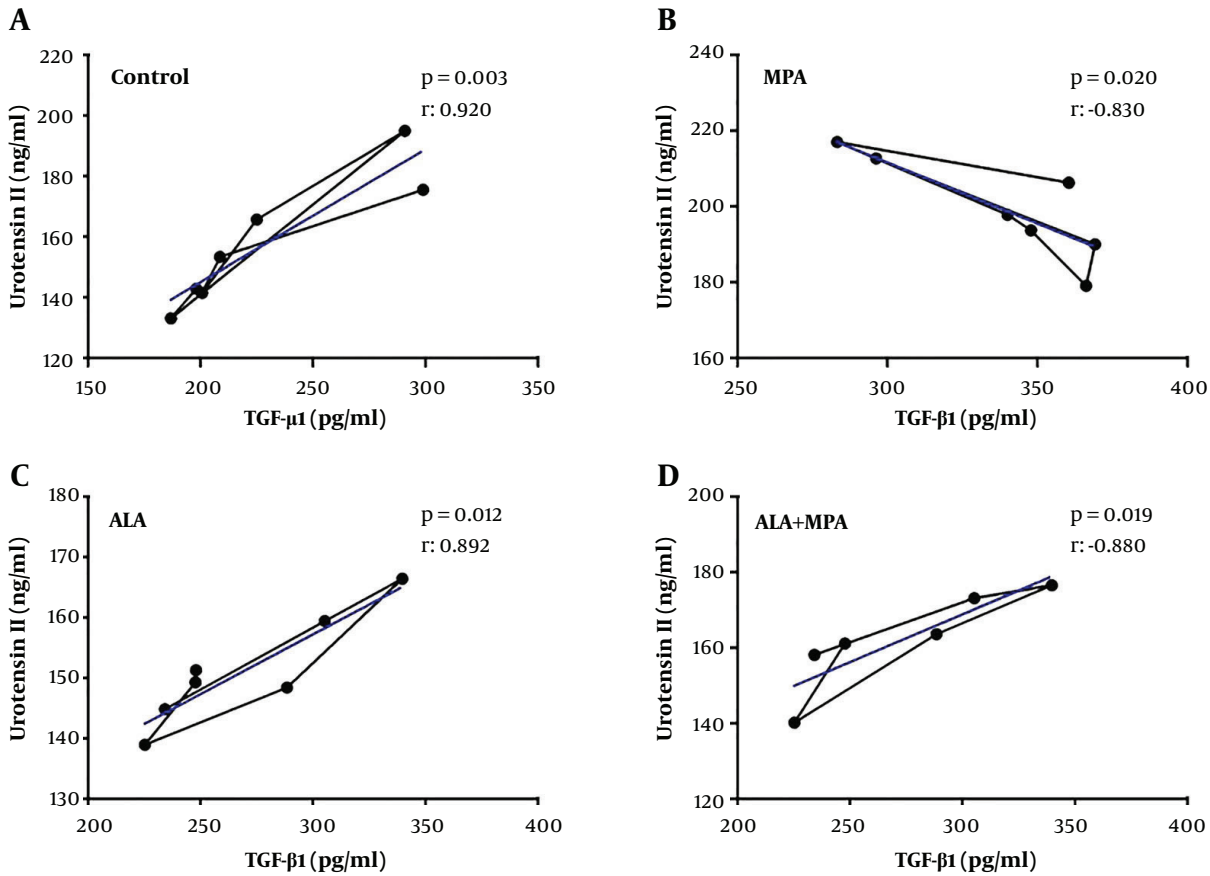

Figure 4. A, The correlation between TGF-β1 and U-II protein levels in control (r = 0.920, P = 0.003); B, MPA (r = -0.830, P = 0.02); C, ALA (r = 0.892, P = 0.012); D, and MPA + ALA (r = 0.884, P = 0.019) groups. MPA, methylprednisolone acetate; ALA, alpha lipoic acid.

by the samples harvested from healthy and osteonecrotic donors and differential effects of GCs based on the status of pathophysiology (46).

Previous studies with different GC-induced ON animal models have shown that treatment with anticoagulant and/or lipid-lowering molecules prevents ON progression (47-49). In another rabbit ON model, GC-treated animals developed oxidative injury of DNA just before the ON onset (50). In line with this finding, an in-vitro model demonstrated that GC treatment of osteoblasts downregulated the expression of bone formation and remodeling factor Cbfa1, resulting in osteoblast dysfunction and apoptosis caused by oxidative stress (51, 52). Moreover, it was observed that antioxidant treatments in GC-induced ON models improved the bone metabolism and rehabilitated oxidative-stress induced ON (51, 53). Weinstein propose that the high levels of osteoblast and osteocyte apoptosis is the leading cause of osteonecrosis, which cause the impaired osseous perfusion due to reduced amounts of angiogenic factors (52).

In the present study, we showed the effects of ALA as an anti-inflammatory and anti-oxidative agent on GC-induced ON in rats. The histopathological examinations revealed significant reduction in both fatty degeneration and ON levels (Table 1, Figure 4). Furthermore, ALA treatment also attenuated oxidative stress levels through reducing both 8-OHdG- and 4-HNE-positive cells in femoral head, which represent cellular DNA damage and lipid peroxidation levels, respectively (Table 1, Figure 3). Similarly, in a rabbit model of GC-induced osteonecrosis, ALA injection significantly reduced ON pathogeny through ameliorating the vascular blood flow and inhibiting the oxidative stress (54). Using its anti-oxidative action mechanism, ALA has been tested as a therapeutic option in the treatment of several diseases such as autoimmune diseases, diabetes mellitus, neurodegenerative disorders, heart diseases, and cancer (32, 33). In order to elucidate the underlying mechanisms through which ALA exert its protective effects, we analyzed the pro-

tein U-II and TGF-β1 levels using ELISA. As depicted in Figure 1, ALA treatment significantly elevated after MPA treatment. In addition, as shown in Figure 2, a strong correlation was observed between U-II and TGF-β1 protein levels in the control and ALA-only treated groups. In contrary, MPA treatment reversed this positive correlation and disturbed the balance between U-II and TGF-β1 protein levels. On the other hand, ALA treatment reconstituted such a strong positive correlation between U-II and TGF-β1 protein levels in MPA + ALA group. In line with our finding, U-II upregulation is closely associated with and a triggering upstream for TGF-β1 pathway (24). Moreover, the increased expressions of both U-II and TGF-β1 proteins in the synovial fluids of OA and osteoradionecrosis patients reflect their pivotal roles in the pathophysiology of such diseases, as shown in our ON animal model (25, 28).

According to the findings in the literature, although TGF-β1 signaling pathway is a prerequisite for the joint construction, its expression later adult stages is implicated in particular degenerative disorders such as OA and ON via synovial fibrosis (55, 56). It is also suggested that, promising novel therapeutic interventions can be emerged for the treatment of osteo-degenerative diseases through modulating or inhibiting the expression of one of the down- or upstream molecules of TGF-β1 signaling pathway (e.g. U-II) (57, 58).

5.1. Conclusions

The present study was to explore the therapeutic effects of ALA, as an anti-inflammatory and anti-oxidative molecule, in GC-induced osteonecrotic rats. To the best of our knowledge, this is the first study showing the ameliorative effects of ALA on ON through inducing a correlative reduction in the expression levels of U-II and TGF-β1 proteins in the rats' femoral head region. Further studies are recommended to detect the connection between ALA action and U-II-TGF-β1 signaling pathways at molecular level and to find out therapeutic potential of this approach in ON patients.

Footnotes

Authors' Contribution: Study concept and design: Recep Dokuyucu. Analysis and interpretation of data: Zeynel Abidin Tas, Hasan Gokce, and Oguzhan Ozcan. Drafting of the manuscript: Irfan Koca.

Conflict of Interests: The authors declare that they have no potential conflict(s) of interest.

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