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Protective effects of alpha-lipoic acid on bleomycin-induced skin fibrosis through the repression of NADPH Oxidase 4 and TGF-β1/Smad3 signaling pathways

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Abstract

The aim of this study was to determine the protective effects of alpha-lipoic acid (ALA), which is known as a powerful antioxidant, and the possible related molecular mechanisms that mediate its favorable action on skin fibrosis in the bleomycin (BLM)-induced scleroderma (SSc) model in mice. The experimental design was established with four groups of eight mice: Control, ALA (100 mg/kg), BLM (5 μg/kg), and BLM + ALA group. BLM was administered via subcutaneous (*sc*) once a day while ALA was injected intraperitoneally (*ip*) twice a week for 21 days. Histopathological and biochemical analyses showed that ALA significantly reduced BLM-induced dermal thickness, inflammation score, and mRNA expression of tumor necrosis factor-alpha (TNF-α) in the skin. Besides, the mRNA expressions of the subunits of NADPH oxidase, which are Nox4 and p22phox, were found to be significantly induced in the BLM group. However, ALA significantly reduced their mRNA expression, which were in parallel to its decreasing effect on serum total oxidant status (TOS) level. Moreover, it was found that ALA downregulated the mRNA expressions of alpha-smooth muscle actin (α-SMA), collagen type I and fibronectin in the skin tissue of the BLM group. Additionally, it was shown that ALA reduced significantly the TGF-β1 and p-Smad3 protein expressions in the BLM + ALA group. On the other hand, ALA did not exhibit any significant effect on the p38 mitogen-activated kinase (MAPK) activation induced by BLM. All these findings point out that ALA may be a promising treatment for the attenuation of skin fibrosis in SSc patients.

Keywords

alpha-lipoic acid, oxidative stress, scleroderma, skin fibrosis

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Introduction

Systemic sclerosis (SSc, scleroderma) is a connective tissue disorder manifested by vascular abnormalities, activation of the immune system, and development of fibrosis in the visceral organs and skin. The pathological hallmark of tissue fibrosis is the excessive production of extracellular matrix (ECM) proteins, such as collagen type 1 and fibronectin.[1,2] Although the exact molecular mechanism underlying the fibrotic process in SSc remains unknown, numerous studies showed that transforming growth factor (TGF)-β displayed a major role as the most potent and profibrogenic cytokine by inducing a phenotypic change of fibroblasts to myofibroblasts, which are more efficient cells in ECM synthesis.[1–4] Also, it has been well known that excessive reactive oxygen species (ROS) resulted by oxidative stress can trigger both expression and activation of TGF-β, which in turn activates the fibrosis process.[3–9] TGF-β signaling was reported mainly to induce profibrotic gene expression by activating its downstream pathway, Smad2/3 pathway. Besides, it was shown that TGF-β could also stimulate p38 mitogen-activated protein kinase (MAPK), which can enhance the collagen production related to tissue fibrosis of SSc.[3–5]

Although there are many endogenous sources of ROS, it has been shown that NADPH oxidases (Nox) are responsible for most ROS generation. The Nox family has seven isoforms: Nox1–5, Duox1, and Duox2. All isoforms, except for Nox3, were demonstrated to express in fibroblasts from different organs such as blood vessels, lungs, kidneys and heart. Among them, Nox4 is different from others in terms of being constitutively active in the presence of its docking subunit, p22phox. Nox4–dependent generation of ROS has been known to be associated with the fibrotic process in SSc.[7–13] A previous study demonstrated that a ROS-mediated loop maintained increased expression of Nox4 in skin fibroblast from patients with SSc.[13] Another study showed that genetic and pharmacological inhibition of Nox4 led to reduced ROS and collagen type I levels in the SSc-cultured fibroblast.[14] Although the regulation mechanism of Nox4 is complex, TGF-β through the Smad2/3 pathway was reported to induce Nox4.[11,14,15] In fact, it has been reported that there is a link between the ROS, which is generated by Nox4, and TGF-β/Smad pathway in a feed-forward manner.[11] In an experimental skin fibrosis model, it was shown that TGF-β could upregulate Nox4 gene expression and activity in a dose and time dependent in human dermal fibroblasts. In the same study, it was also reported that Nox4 knockdown by siRNA led to decrease alpha-smooth muscle actin (α-SMA), collagen type I, and fibronectin synthesis in the skin.[15]

In this regard, accumulating evidence has also drawn attention to the beneficial effects of various antioxidant agents on skin fibrosis by suppressing oxidative stress in the experimental SSc models.[16–20]

Alpha-lipoic acid (ALA), known as thioctic acid, is a powerful antioxidant with its both water and fat soluble properties. It exhibits antioxidant action by scavenging ROS, chelating metals and regenerating endogenous antioxidants. It is also used clinically for patients with diabetic neuropathy.[21] Moreover, it was reported that ALA had a beneficial role against cardiac,[22] liver[23–25], and pulmonary fibrosis[26] in experimental models. Interestingly, it was shown that the levels of intracellular ALA and the lipoic acid synthetase enzyme, which is responsible for ALA synthesis, were lower in the dermal fibroblasts isolated from SSc patient skin than that of healthy subjects. In addition, dihydrolipoic acid, which is an active metabolite of ALA, was shown to prevent the myofibroblast differentiation of SSc dermal fibroblasts, which in turn led to decreased expression of α-SMA, collagen type I, and platelet-derived growth factor (PDGF).[27] However, the molecular mechanisms of ALA against skin fibrosis have not been reported. Therefore, this study was performed to determine the protective effects of ALA and the possible related molecular mechanisms which mediate its favorable action on skin fibrosis in a bleomycin (BLM)-induced SSc model in mice.

Materials and methods

Animals and study design

Our study was carried out with the approval of Dokuz Eylul University Animal Ethics Committee (31/2018). Thirty-two Balb/c mice (6–8-week-old; weighing 20–22 g) were used and all animals were maintained under standard conditions (20–22°C, 55%–60% humidity and 12-h light-dark cycle).

The BLM-induced skin fibrosis model was achieved as described previously.[28] The animals were separated randomly into four groups of eight: The control group was injected isotonic saline (Biofleks, Istanbul, Turkey) via subcutaneous *(sc)* once a day and intraperitoneal *(ip)* injections twice a week for 21 days. The ALA group was given isotonic saline via *sc* once a day and 100 mg/kg ALA (Thioctacid T flacon, MEDA Pharma, Hamburg, Germany) by *ip* injections twice a week for 21 days. The BLM group was given 5 µg/kg BLM (Onko Kocsel, Kocaeli, Turkey) by *sc* once a day and isotonic saline by *ip* twice a week for 21 days. The BLM + ALA group was injected with 5 µg/kg BLM by *sc* once a day and 100 mg/kg ALA by *ip* twice a week for 21 days. The dosage of ALA used in this study was chosen by considering the safety profile of ALA[29] and its ability to protect from marked oxidative damage in other experimental models.[30–31] Sacrification was performed under ether anesthesia on 21 days. The injected skin section was taken for histopathological and biochemical analyses. Additionally, blood samples were taken from the *vena cava caudalis* for evaluating serum parameters.

Histopathological examination

Masson's trichrome (Agilent Technologies, Dako, USA) staining was done to study the dermal thickness of the sections of skin tissues of mice. The dermal thickness was measured at 10 randomly selected areas by using an image analyzer (Nikon, Nikon NIS-Elements, Japan) including a microscope (Nikon Eclipse C1, Japan) and a camera (Nikon DS-Fi2, Japan) at 200-fold magnification. Also, all sections were stained with hematoxylin-eosin (H&E) (Agilent Technologies, Dako, USA) for the assessment of inflammation. The inflammation was scored semiquantitavely as: 0 = normal, 1 = mild, 2 = moderate, and 3 = severe, respectively, indicating that no inflammatory cells, few inflammatory cells, and extensive inflammatory cells.[32]

Immunohistochemical analysis

α-SMA protein expression in the skin tissue was evaluated immunohistochemically. Following blocking, α-SMA primary antibody diluted 1:100 (Catalog number: 5,268,303,001, NexES, Roche USA) and secondary antibody (Thermo, USA) were applied, respectively, on the deparaffinized and rehydrated skin tissue sections. Then, 3, 3*ı*-diaminobenzidine (DAB) (Agilent Technologies, Dako, USA) staining and Mayer hematoxylin (Agilent Technologies, Dako, USA) counterstaining were done. Finally, α-SMA positive cells were counted by using a microscope (Nikon Eclipse C1, Japan) and camera (Nikon DS-Fi2, Japan) at 200-fold magnification.

Determination of serum total oxidant status (TOS) and total antioxidant status (TAS) levels

To evaluate systemic oxidative stress, serum TOS and TAS levels were analyzed in the experimental groups with colorimetric kits (Rel Assay, Turkey). The calibration curve was constructed with hydrogen peroxide (H_2O_2) for TOS assay. The results were expressed as μmol H_2O_2 Equivalent/L.[33] For the measurement of the serum TAS levels, a Trolox equivalent, which is a vitamin E analog, was used as a standard and the results were presented as mmol Trolox Equivalent/L.[34]

Preparation of skin tissue for biochemical analyses

First, mice skin samples were homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) by using tissue lyser (Qiagen, Hilden, Germany). Then, total RNA and protein were isolated simultaneously from the homogenates.[35]

Real time quantitative polymerase chain reaction (qPCR)

First, cDNA synthesis was achieved from isolated RNA by using a reverse transcription kit (Prime Script RT, Qiagen, USA). Then, qPCR was established with SYBR Green PCR master mix (RT^2 SYBR Green qPCR Mastermix, Qiagen, USA) using the qPCR instrument (Rotor-Gene, Qiagen, USA). The primer sequences (Oligomer Biotecnology, Turkey) are presented in Table 1 ß actin, which is a housekeeping gene, was used for the normalization of the results. Gene expression fold changes were calculated according to the $2^{-\Delta\Delta}$ Ct method.[36]

Western blot

Skin tissue homogenates (30 μg protein per well) were run on 12% sodium dodecyl sulfate polyacrylamide gels. Then, they were transferred onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, USA) by using a blotting system (BioRad, USA).[37] Following blocking with 5% nonfat dry milk, membranes were subjected to primary antibodies of TGF-β1 (1:750, Abcam Technology, Cambridge, UK), phospho-Smad2 (p-Smad2) (Ser465/467) (1:1000, Cell Signaling Technology Inc, Danvers, MA, USA), p-Smad3 (Ser423/425) (1:1000, Cell Signaling Technology Inc, Danvers, MA, USA), and p-p38 MAPK (1:1000, Cell Signaling Technology Inc, Danvers, MA, USA). After washing steps, membranes were incubated with secondary antibody (1:1000, Cell Signaling Technology Inc, Danvers, MA, USA). Proteins bands were visualized with ECL

Table 1. Mouse qPCR primers.

Gene	Forward primers	Reverse primers
α-SMA	GAGGCACCACTGAACCCTAA	CATCTCCAGAGTCCAGCACA
Collagen type I	ATCTCCTGGTGCTGATGGAC	ACCTTGTTTGCCAGGTTCAC
Fibronectin	CGAGGTGACAGAGACCACAA	CTGGAGTCAAGCCAGACACA
Nox4	CCCAAGTTCCAAGCTCATTTCC	TGGTGACAGGTTTGTTGCTCCT
p22phox	GGAGCGATGTGGACAGAAGTA	GGTTTAGGCTCAATGGGAGTC
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
β-actin	AGGCATCCTCACCCTGAAGTA	CACACGCAGCTCATTGTAGA

reagent (Millipore, Bedford, USA). Then, stripping was done to reprobe the membranes with the primary antibodies of total Smad2/3 (1:1000, Cell Signaling Technology Inc, Danvers, MA, USA) and total p38 MAPK (1:1000, Cell Signaling Technology Inc, Danvers, MA, USA) in order to determine the ratio of phosphorylated protein to its total pair. ß actin (1:2000, Cell Signaling Technology Inc, Danvers, MA, USA) was used for the normalization of TGF-β1. Band densities were evaluated by using Image-J software (1.8.0_271) (National Institute of Health, USA).[38]

Protein assay

Protein contents of the skin tissue homogenates were detected by BCA assay (Biovision, Milpitas, CA, USA).[39]

Statistical analysis

All statistical analyses were done by Prism version 8.0 (GraphPad Software, California, US). The Kruskal–Wallis test was performed to determine the differences between multiple independent groups. The Mann–Whitney *U* test was used to analyze the difference between two independent groups. Pearson correlation test was done to determine relationships between Nox4 and p-Smad3, which were normally distributed. The relationships between TGF-β and p-p38 MAPK protein levels, which did not exhibit normal distribution, were analyzed by the Spearman correlation test. All data were presented as mean ± SEM. $p < 0.05$ was considered statistically significant.

Results

ALA suppressed the BLM-induced skin fibrosis in mice

Masson's trichrome staining demonstrated that BLM increased significantly the dermal thickness in comparison to that in the control group ($p < 0.05$). However, ALA administration significantly decreased the dermal thickness in the BLM + ALA group ($p < 0.05$), which reflected to decreased collagen deposits in the skin tissue (Figure 1). Also, it was found that there was a significant increase in the α-SMA positive myofibroblasts in the lesional skin of mice in the BLM group in comparison to the control ($p < 0.001$). However, ALA significantly decreased the number of these cells ($p < 0.05$) (Figure 2(a) and (b)). In parallel to this finding, the mRNA expression of α-SMA in the skin was significantly upregulated by 64.7 fold in the BLM group ($p < 0.001$). However, ALA significantly reduced its mRNA expression by 10.26 fold in the BLM + ALA group ($p < 0.05$) (Figure 2(c)). Moreover, the mRNA expressions of collagen type I and fibronectin in the skin tissue were found to be significantly

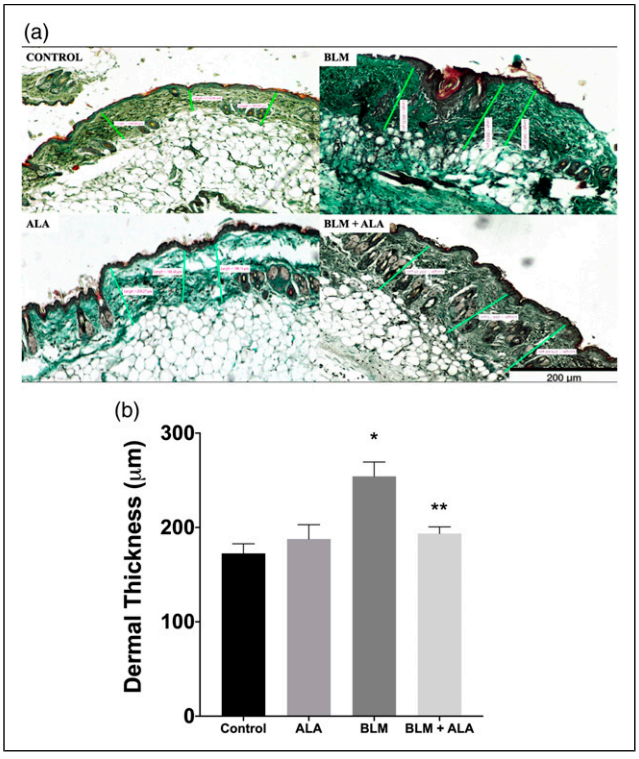

Figure 1. (a) Representative images of Masson's trichrome stained skin tissue of the experimental groups. (b) The graph showing the dermal thickness. Data are presented as mean ± SEM *$p < 0.05$ vs control, **$p < 0.05$ vs BLM.

elevated by 7.28 and 4.33 fold, respectively, in the BLM group ($p < 0.05$ and $p < 0.05$, respectively). However, ALA significantly suppressed their mRNA expressions by 0.94 and 0.97 fold, respectively, in the BLM + ALA group ($p < 0.05$ and $p < 0.05$, respectively) (Figures 2(d) and (e)).

ALA decreased oxidative stress in the BLM-induced fibrotic skin of mice

Serum TOS level was detected to be significantly higher in the BLM group (14.66 ± 2.40 µmol/L) compared to that of the control group (7.66 ± 0.83 µmol/L) ($p < 0.05$). However, ALA administration significantly decreased serum TOS level (9.2 ± 1.75 µmol/L) ($p < 0.05$) (Figure 3(a)). Although there were changes in the serum TAS level, they were not found to be statistically significant (Figure 3(b)).

Additionally, the mRNA expressions of Nox4 and p22phox were analyzed to evaluate the impact of ALA on oxidative stress in the skin tissue of experimental groups. It was found that the mRNA expressions of Nox4 and p22phox were significantly elevated by 7.05 and 5.08 fold in the BLM group ($p < 0.05$ and $p < 0.001$, respectively). However, ALA significantly decreased the mRNA expressions of Nox4 and p22phox to 1.09 and 2.65 fold in the

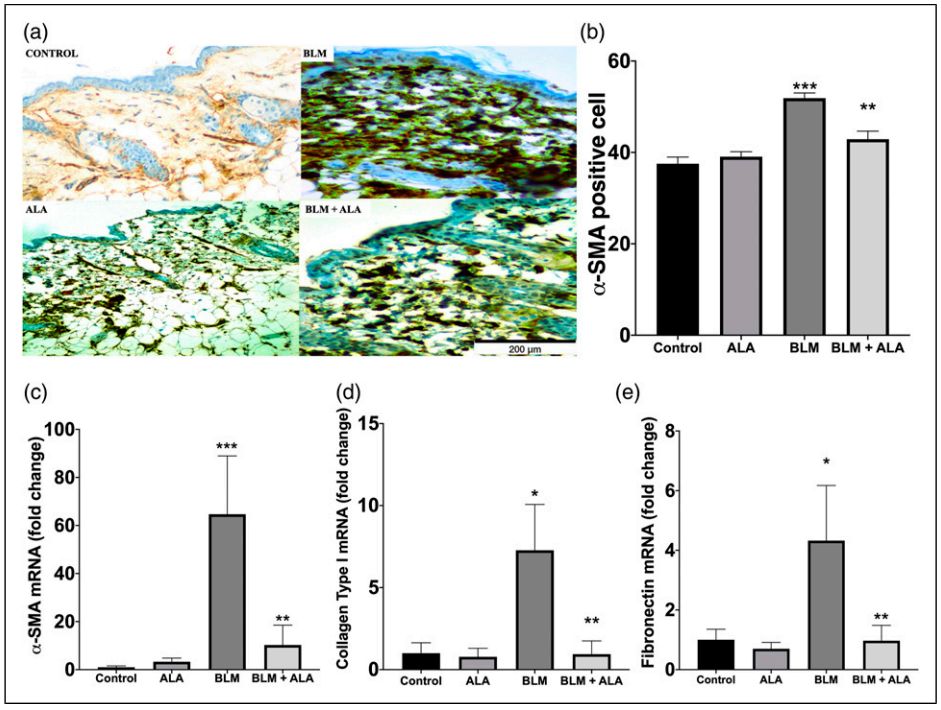

Figure 2. (a) α-SMA immunoreactivity. (b) The graph showing the number of α-SMA positive cells. (c) mRNA expressions of α-SMA, (d) collagen type I, and (e) fibronectin in the skin of the experimental groups. Data are presented as mean ± SEM ***$p < 0.001$ and *$p < 0.05$ vs control, **$p < 0.05$ vs BLM.

BLM + ALA group ($p < 0.05$ and $p < 0.05$, respectively) (Figure 3(c) and (d), respectively).

ALA suppressed the number of inflammatory cells and mRNA expression of TNF-α in the BLM-induced fibrotic skin of mice

Compared with the control group, inflammatory cells were determined to be significantly higher in the BLM group ($p < 0.05$). However, ALA decreased the number of these cells in the skin ($p < 0.05$ (Figures 4(a) and (b)). Also, it was found that BLM induced its mRNA expression by 5.43 fold in the skin tissue of mice ($p < 0.05$). However, ALA reduced the mRNA expression of TNF-α to 2.05 fold in the BLM + ALA group ($p < 0.05$) (Figure 4(c)).

ALA suppressed TGF-β1/Smad3 signaling in the BLM-induced fibrotic skin of mice

TGF-β1 protein expression in the skin was found to be significantly higher in the BLM group compared to the control group ($p < 0.001$). However, ALA led to a significant decline in the TGF-β1 protein expression in the BLM + ALA group ($p < 0.05$) (Figure 5(a)). Besides, Smad2/3 activations were evaluated by p-Smad2 and p-Smad3 protein expression. Compared to the control, there

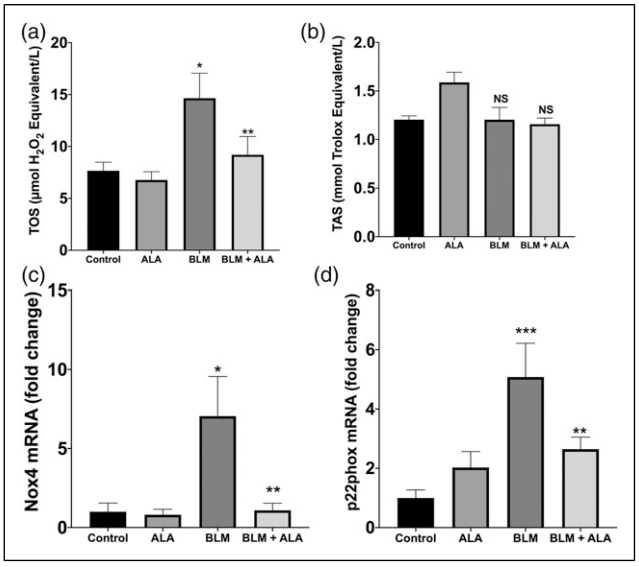

Figure 3. (a) Serum TOS and (b) TAS levels. (c) mRNA expression of Nox4 and (d) p22phox in the skin of the experimental groups. Data are presented as mean ± SEM ***$p < 0.001$ and *$p < 0.05$ vs control, **$p < 0.05$ vs BLM, NS: not significant.

were significant increases in their protein expression in the skin of the BLM group ($p < 0.05$ and $p < 0.05$, respectively). Although there were decreases in the p-Smad2 and p-Smad3

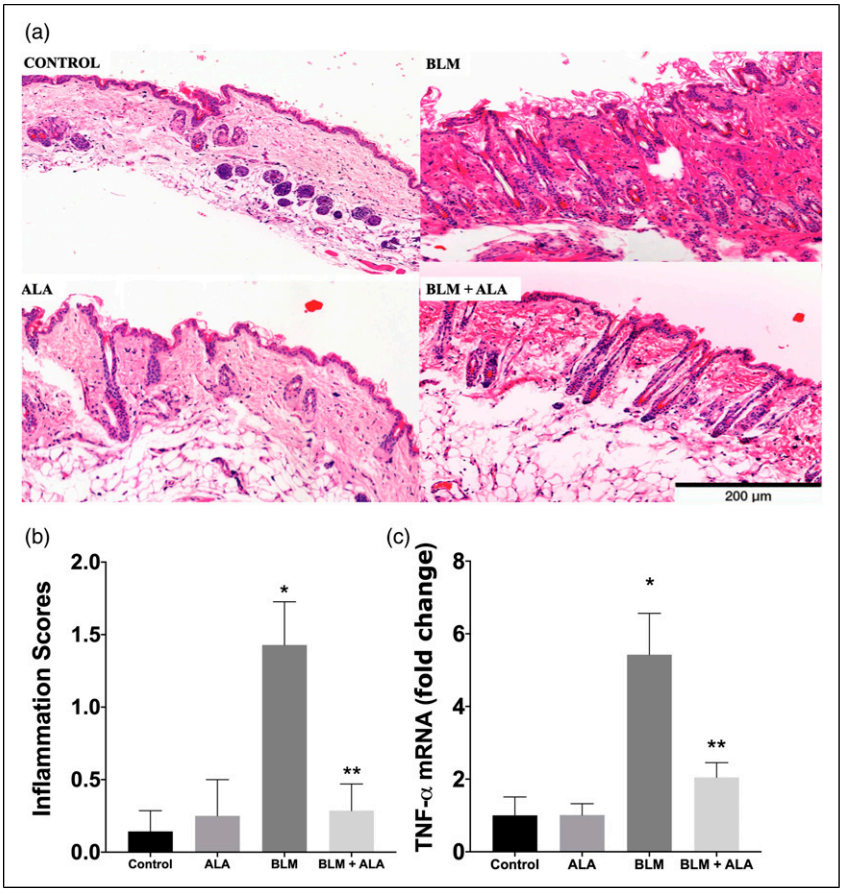

Figure 4. (a) Representative images of H-E stained skin tissue of the experimental groups. (b) The graph showing the inflammation score. (c) mRNA expression of TNF-α. Data are presented as mean ± SEM $*p < 0.05$ vs control, $**p < 0.05$ vs BLM.

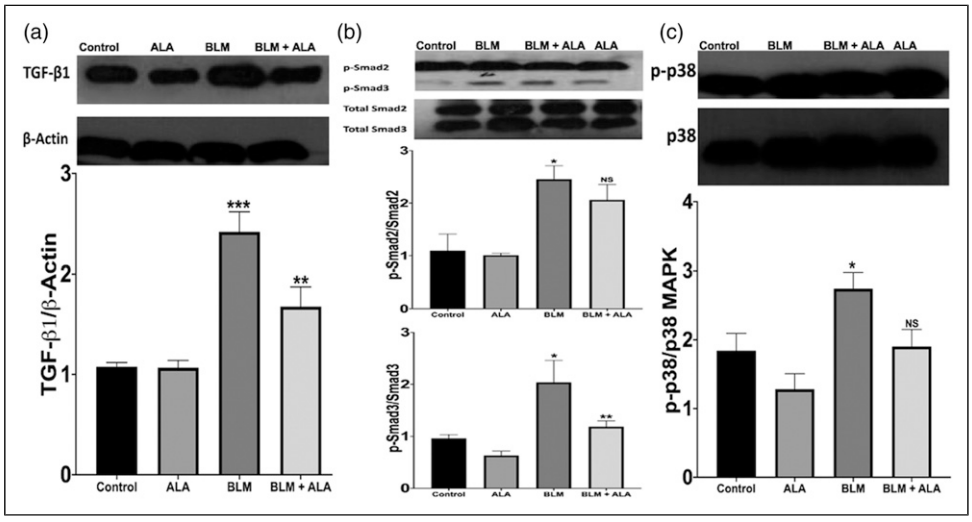

Figure 5. Representative Western blot images and graphics representing densitometric analysis of the protein bands. (a) Skin tissue protein expression of the TGF-β1, (b) p-Smad2, total Smad2, p-Smad3 and total Smad3 and (c) p-p38 MAPK and total p38 MAPK of the experimental groups. Data are presented as mean ± SEM $***p < 0.001$ and $*p < 0.05$ vs control, $**p < 0.05$ vs BLM, NS: not significant.

protein expressions of the BLM + ALA group, the change only in the p-Smad3 was determined to be statistically significant ($p < 0.05$) (Figure 5(b)). Additionally, it was found that there was a positive correlation between p-Smad3 and Nox4 (r = 0.42, $p = 0.033$).

ALA did not have any effect on p38 MAPK signaling in the BLM-induced fibrotic skin of mice

P-p38 MAPK protein expression, which is the active form of p38 MAPK, in the skin was found to have significantly induced in the BLM group ($p < 0.05$). Although there was a decreasing in the p-p38 MAPK of the BLM + ALA group, the difference was not found to be a statistical significance ($p > 0.05$) (Figure 5(c)). On the other hand, p-p38 MAPK was found to be positively correlated with TGF-β1 (r = 0.46, $p = 0.014$).

Discussion

This study presents that ALA has a favorable action on skin fibrosis in the BLM-induced SSc model in mice, which was confirmed by the decreased oxidative stress, inflammation and fibrosis. This ability of ALA is at least partially related to the repression of Nox4 and TGF-β1/Smad3 signaling pathways (Figure 6).

In this study, the histopathological examination showed that the administration of BLM, which was known to induce fibrosis by enhancing oxidative stress,[40] resulted in a significant increase in the dermal thickness and expression of α-SMA, a strong marker for myofibroblast differentiation. In parallel to these findings, the mRNA expression of collagen type I and fibronectin were found to be significantly increased in the BLM administered mice, confirming that the BLM-induced skin fibrosis in SSc model was successfully established. However, ALA administration two times in a week followed by BLM led to significant improvements in those parameters.

As previously stated, oxidative stress has a critical role in the underlying molecular mechanism of the SSc.[6–8] Therefore, we hypothesized that ALA could exhibit an antifibrotic effect through its antioxidant ability. In this direction, we first analyzed TOS level in the serum to assess the systemic oxidative stress among the experimental groups. It was detected that there was a significant elevation in the TOS level of the BLM administered group in comparison to the control group, which agreed with the previous reports.[7,8] However, ALA significantly reversed this change. In contrast, there were no statistically significant changes among groups in terms of serum TAS levels in this study. With respect to this finding, conflicting results exist in the literature, which demonstrate that there could be significant[6] or insignificant changes of the serum TAS levels.[41] At this point, it might be thought that a notable imbalance arose from oxidative stress and the relatively insufficient antioxidant status in the experimental groups of this study. Also, this finding provided a confirmation to demonstrate

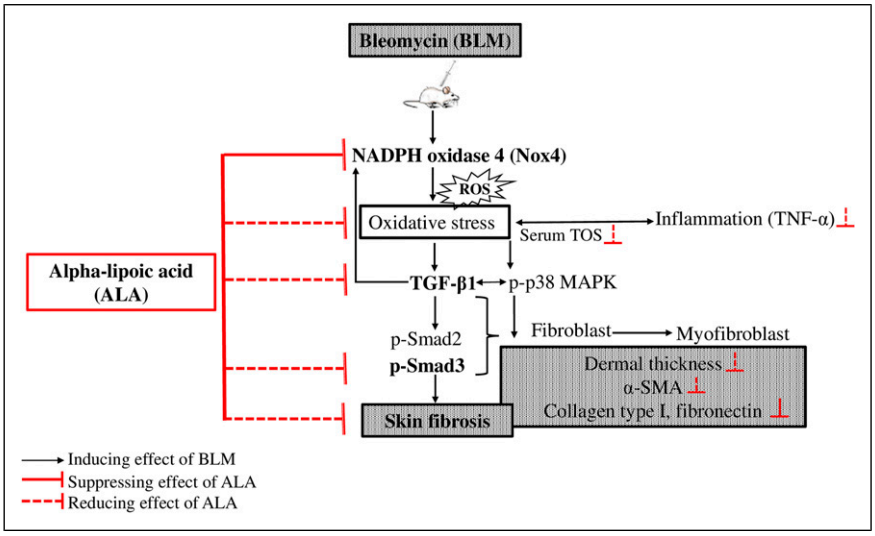

Figure 6. A schematic overview of the possible protective effects of alpha-lipoic acid (ALA) on bleomycin-induced (BLM) skin fibrosis in mice. In the BLM-induced skin fibrosis, NADPH oxidase 4 (Nox4) might induce, resulting in oxidative stress by excessive production of ROS. Oxidative stress and inflammation can stimulate each other and also subsequently induce TGF-β1/Smads and p38 MAPK pathways, which in turn result in fibrosis process, meaning that increases in the dermal thickness and production of α-SMA, collagen type I and fibronectin. By the way, TGF-β1 could upregulate Nox4. The protective effects of ALA against BLM-induced skin fibrosis might be partially related to the repression of Nox4 and TGF-β1/Smad3 signaling pathways.

the strong association between the profoundly oxidative stress and skin fibrosis induced by BLM.

Alternatively, studies have reported that especially, Nox4, an isoform of NADPH oxidase enzyme family, exhibits an important role as a source of ROS formation, which is a main player for fibrotic process in SSc.[7–15] Based on those studies, we next investigated whether ALA affected Nox4 regulation in this study. Our results showed that the Nox4 and p22phox mRNA expressions were significantly induced in the BLM group. However, ALA was markedly reduced the mRNA expressions of Nox4 and p22phox, which were also in line with the decreased serum TOS level. A previous study conducted on liver fibrosis induced by concanavalin-A, which is except for skin fibrosis induced by BLM, demonstrated that ALA could downregulate the expressions of Nox1 and also Nox4 isoform.[25] By the way, oxidative stress and inflammation have been well known to stimulate one another.[7] In this direction, we also found that there was a significant induction in the TNF-α mRNA expression of the BLM group. However, ALA could significantly decrease the mRNA expression of TNF-α. Consistent with this finding, the inflammation score, which was evaluated histopathologically, was found to be significantly declined in the ALA administered BLM group, indicating that the anti-inflammatory property of ALA. However, the anti-inflammatory effect of ALA should not be restricted to those findings. It requires additional evaluation with a powerful inflammation indicator, such as myeloperoxidase activity.

On the other hand, previous studies reported that TGF-β, which is the most potent profibrotic cytokine, was overexpressed in all fibrotic tissues and played an important role in the regulation of Nox4, a major enzymatic source for ROS.[7–15] In fact, the activation of TGF-β by ROS results in the interaction between TGF-β and its receptor, TβR2, which in turn bind to TβR1 to activate the downstream Smad2/3 pathway. It was known that especially, activation of Smad3 played a major role in the expression of the α-SMA gene, which is responsible for the myofibroblast differentiation.[42] Moreover, other previous studies demonstrated that the interruption of TGF-β/Smad3 signaling resulted in attenuated skin fibrosis.[43,44] In this context, we found the significant increased protein expression of TGF-β and activation of Smad2/3 in the BLM group, which were consistent with previous studies.[19,20,45] However, ALA had a significant reducing effect on the TGF-β1 and activation of Smad3, which was reflected to the p-Smad3 protein expression. All these findings suggested that ALA could also affect further regulation of the TGF-β1 activated Smad3 pathway, which in turn led to decreased the mRNA expressions of α-SMA, collagen type I, and fibronectin in the skin tissue. However, the impact of ALA on the total collagen level could not evaluate in this study since the hydroxyproline level, which is used as a marker for total collagen content,[46] was not measured due to the limited lesional skin samples. On the other hand, the role of increased mRNA level of COL1A1, COL1A2, and COL1A3, which are the genes of collagen type I, II, and III, respectively, and also TIMP-1, an endogenous inhibitor responsible for ECM regulation, were demonstrated by a previous study performed on BLM-induced SSc model.[47] Thus, further studies are needed to elucidate the detailed mechanisms of the effects of ALA on the collagen metabolism in the BLM-induced skin fibrosis. However, this is the first study presenting the suppressing effect of ALA on the TGF-β1/Smad3 signaling in BLM-induced skin fibrosis, which is parallel to its reducing effect on Nox4 in mice. Also, a significant positive correlation determined between the Nox4 and activation of Smad3 was in accordance with previous reports emphasizing that there was a relation in a feed-forward manner between Nox4 and TGF-β/Smad2/3 pathway.[11,12,14]

Additionally, it was shown that p38 MAPK, which is also activated by ROS, affected TGF-β induced fibrosis independently from Smad2/3 pathway.[3–5] Also, the activation of p38 MAPK was associated with increased collagen type I and TGF-β in scleroderma fibroblasts. Besides, the suppression of p38 MAPK with its specific inhibitor was reported to result in a significant reduction of the collagen type I, which was stimulated by TGF-β.[48] In contrast, another study reported that p38 MAPK activation was not correlated with collagen production.[49] The reason for different findings in those studies was interpreted as different culture conditions resulted in different findings. In this direction, p38 MAPK activation was determined to be significantly higher in the BLM group than in the control group in this study, which is supported by a previous study conducted on skin fibrosis in the BLM-induced SSc model.[20] Also, a positive significant correlation was found between TGF-β1 and p38 MAPK activation in this study, suggesting that there could be a relation among them. There were changes in the p-p38 MAPK protein expression of the ALA administered BLM group, but they could not reach statistical significance. We have found no study to compare our findings related to ALA's effect on the p38 MAPK pathway.

In conclusion, our findings provide that ALA has notably protective effects on skin fibrosis induced by BLM, at least in part through the repression of Nox4 and TGF-β1/Smad3 signaling pathways, which can reflect its antioxidant, anti-inflammatory, and antifibrotic effects. Therefore, ALA may be a promising treatment for the attenuation of skin fibrosis in SSc patients.

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