

Targeting Oxidative Stress, Transforming Growth Factor Beta-1, and the Mammalian Target of Rapamycin by Valproic Acid to Ameliorate Bleomycin-Induced Scleroderma

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Abstract

Objective: To explore the possible ameliorative effect of valproic acid on the experimental model of skin fibrosis induced by bleomycin. **Materials and Methods:** Forty male BALB/c mice were divided into four equal groups as follows: control group, bleomycin group, bleomycin + Valproic acid group, and Valproic acid group. Mice were assessed for their body weight every 3 days throughout the whole study. Skin tissues were used to evaluate the oxidative stress parameters, transforming growth factor beta 1 (TGF- β 1), tumor necrosis factor alpha, interleukin 15, and mammalian target of rapamycin (mTOR). Skin fibrosis was evaluated by measuring dermal thickness and staining the skin tissues with Masson trichrome stain. Furthermore, the skin tissues were immunostained with alpha smooth muscle actin (α -SMA). **Results:** Administration of Valproic acid to bleomycin-treated mice resulted in the restoration of the body weight with significant decrease in the dermal thickness, amelioration of oxidative stress, suppression of TGF- β 1 and mTOR expression, and significant reduction of the percentage of α -SMA immunostaining and the proinflammatory cytokine levels compared to mice treated with bleomycin alone. **Conclusion:** Valproic acid has an antifibrotic effect on skin fibrosis which may represent a beneficial therapeutic modality for the management of scleroderma.

Keywords: Bleomycin, mice, scleroderma, transforming growth factor beta 1, Valproic acid

INTRODUCTION

Scleroderma or systemic sclerosis is a complex connective tissue disease characterized by fibrosis of the skin and other internal organs in the body such as lung, heart, gastrointestinal tract, and kidney.^[1] Its pathogenesis is still unclear, but many factors may play a role in the disruption of connective tissue structure such as excessive deposition of extracellular matrix that is preceded by autoimmunity, inflammation, and vasculopathy.^[2] Many mediators and pathways are involved in the fibrogenesis process and create a microenvironment which activates fibroblasts to differentiate into myofibroblasts that produce high amount of different types of collagen and secrete a lot of growth factors that exacerbate the ongoing fibrotic process. From these mediators, transforming growth factors β 1 (TGF- β 1) and mammalian target of rapamycin (mTOR) kinase may play an important role in the fibrotic process.^[3]

TGF- β 1 is a central profibrotic cytokine that controls collagen production by triggering profibrotic gene overexpression.^[4] mTOR kinase is considered as the master regulator of phosphoinositide 3-kinase/protein kinase B (AKT) pathway which is linked to cellular metabolism, proliferation, differentiation, and survival.^[5] Any alteration of mTOR signaling may lead to inflammation, cell proliferation, and fibrosis due to the contribution of mTOR signaling to TGF- β 1-induced collagen synthesis.^[6] Hence, it can be considered as an attractive target for the antifibrotic drug testing. Moreover, histone deacetylase has a strong relationship to

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fibrosis and vascular dysfunction through affection of promotor region of collagen-suppressor gene. Trichostatin A, which is a potent histone deacetylase inhibitor, decreased collagen in skin fibroblasts *in vitro* through the suppression of TGF- β 1.^[7]

Till now, the therapeutic modalities of scleroderma are scarce and cause many adverse effects as immunosuppressive drugs. Therefore, the search for new options for amelioration of scleroderma is mandatory. Valproic acid is a well-known drug used in the treatment of many types of epilepsy and known as an inhibitor of histone deacetylase.^[8] Recent studies proved its promising role as an antifibrotic agent in different organs as the lung, peritoneum, liver, and kidney. It was reported that divalproex sodium may be a potential therapy for scleroderma-induced digital ulcer as its administration resulted in remission of the lesions with recurrence after drug discontinuation.^[9] Therefore, the current study was conducted to explore the possible ameliorative effect of Valproic acid on a mouse model of skin fibrosis and to examine the possible mechanisms involved in its protective effects, especially affection of mTOR kinase.

MATERIALS AND METHODS

Animals

Forty male BALB/c mice aged 6–8 weeks old and weighing 20–25 g were obtained from the animal house of Tanta University, Egypt. They were maintained for 2 weeks acclimatization period under the standard laboratory conditions of temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$), relative humidity ($60\% \pm 4\%$), and equal day and night cycle with free access to a standard pellet diet and water *ad libitum*. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U. K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Drugs and chemicals

Bleomycin as a powder vial was purchased from Criticare Laboratories Pvt. Ltd., India. Valproic acid sodium salt 98% was purchased from Sigma-Aldrich Co.(St. Louis, Missouri, USA). All other reagents were obtained from Sigma-Aldrich Co.(St. Louis, Missouri, USA) and Emsure Merck, Germany and were of analytical grade. Valproic acid was dissolved in 0.9% saline.

Experimental design

Body weight was assessed regularly every 3 days throughout the whole study. Skin fibrosis was induced by alternate day subcutaneous injection of 100 μl of bleomycin dissolved in phosphate-buffered saline at a concentration of 0.5 mg/ml for 3 weeks. An area measuring 1 cm^2 was drawn after shaving of the upper dorsum of the skin, and bleomycin was injected subcutaneously at the corners and in the middle of this square.^[10] Mice were randomly allocated into four equal groups as follows:

- Control group ($n = 10$): Mice were injected intraperitoneally with 0.5 ml 0.9% saline daily for 21 days.
- Bleomycin group ($n = 10$): Mice were injected subcutaneously with 100 μl of bleomycin on alternate days for 21 days^[10]
- Bleomycin + Valproic acid group ($n = 10$): Mice were injected with bleomycin as bleomycin group concomitantly with Valproic acid intraperitoneally in a dose of 100 mg/kg/day for 21 days.^[11]
- Valproic acid group ($n = 10$): Mice were injected intraperitoneally with Valproic acid in a dose of 100 mg/kg/day for 21 days.

Animal sacrifice and samples collection

Three mice died during the whole period of the study, two of them died at the 15th day from bleomycin group and one at the 10th day from valproic acid group. On day 22 of the study, mice were weighed and euthanized through cervical dislocation after diethyl ether anesthesia. For every mouse, the marked skin area was excised, washed with phosphate-buffered saline and divided into two parts; one was fixed in 10% neutral-buffered formaldehyde for histopathological examination, and the other was divided and frozen immediately at -80°C for further assay of the biochemical parameters.

Determination of tissue oxidative stress parameters

Skin tissues were minced and homogenized with the aid of polytron homogenizer in 50 mM potassium phosphate pH 7.5 for malondialdehyde (MDA) assay, and in 50 mM potassium phosphate pH 7.5, 1 mM ethylenediaminetetraacetic acid for reduced glutathione (GSH) and glutathione-S-transferase (GST) assay at a ratio of 5 ml for each gram tissue. Tissue levels of MDA and GSH were assayed by colorimetric methods according to Ohkawa *et al.*^[12] and Beutler *et al.*,^[13] respectively, using kits produced by Biodiagnostics Company, Egypt (Catalog No. MD2529 and GR2511, respectively). Tissue GST was measured using ELISA kits purchased from MyBioSource, Inc., San Diego, CA, USA (Catalog No. MBS2608156) according to the manufacturer's protocol.

Determination of tissue tumor necrosis factor alpha, transforming growth factor beta 1 and interleukin 15

Skin tissues were minced and homogenized in phosphate-buffered saline at a ratio of 9 mL for each gram tissue with the aid of polytron homogenizer. Then, the homogenates were centrifuged at 4000 rpm for 10 min to get the supernatant. Assay of transforming growth factor beta 1 (TGF- β 1) was carried out using commercially available ELISA kits obtained from Cloud-Clone Corp, USA (Catalog No. SEA124Mu) according to the manufacturer's instructions. Tumor necrosis factor alpha (TNF- α) was determined using ELISA kits purchased from RayBiotech, Inc., USA (Catalog No. ELM-TNFa) according to the manufacturer's protocol. Tissue interleukin 15 (IL-15) was measured using ELISA kits obtained from Abcam, USA (Catalog No.: ab100701) according to the instructions of the manufacturer.

Determination of mammalian target of rapamycin (mTOR)

Assay of tissue mTOR was done using commercially available ELISA kits obtained from Cloud-Clone

Corp, USA (Catalog No. SEA806Mu) according to the manufacturer's protocol.

Histopathological evaluation

Skin tissues from each mouse were fixed in 10% neutral-buffered formaldehyde and embedded in paraffin. Sections of 4 μ m thickness were stained with hematoxylin and eosin and Masson trichrome (MT) stains and examined under the light microscope for evaluation of skin fibrosis. Dermal thickness was measured in each slide with Image J software 1.52a, national institute of health, Bethesda, Maryland, USA using scale line calibration.

Immunohistochemical evaluation

Sections from the skin were immunostained with primary antibody polyclonal IgG to mouse alpha smooth muscle actin (α -SMA) (Affinity biosciences, ltd, Catalog No. AF1032) and the slides were observed under light microscope. Calculation of the percentage of positive immunostaining was done through image J software using IHC profiler plugin for 5 random fields from each slide magnification ($\times 200$).^[14]

Statistical analysis

Statistical analysis of the results was performed using Rstudio Version 1.1.447– © 2009–2018 RStudio, Inc. Data were expressed as mean $\pm$ standard error of the mean. Multiple comparisons were performed using the one-way analysis of variance (ANOVA) followed by *post hoc* Tukey test. The 0.05 level of probability was used as a criterion of significance.

RESULTS

Effects of bleomycin and Valproic acid on the body weight

Bleomycin induced significant decrease in the body weight compared to the control group ($P < 0.05$). Administration of Valproic acid to bleomycin-induced scleroderma mice induced significant increase in the body weight compared to bleomycin group ($P < 0.05$). Valproic acid alone induced nonsignificant effect on the body weight compared to the control group ($P > 0.05$) [Figure 1].

Effect of bleomycin and valproic acid on oxidative stress parameters

Bleomycin induced significant increase in tissue MDA and significant decrease in tissue GSH and GST compared to the control group ($P < 0.05$). Administration of valproic acid concomitantly with bleomycin resulted in significant reduction of MDA and significant increase in GSH and GST in the skin tissues compared to bleomycin group ($P < 0.05$). Administration of Valproic acid alone induced nonsignificant effect on the above-mentioned parameters compared to the control group ($P > 0.05$) [Table 1].

Effect of bleomycin and Valproic acid on tissue TGF- β 1, IL-15, and TNF- α

Bleomycin induced significant increase in tissue TGF- β 1, IL-15, and TNF- α compared to the control group ($P < 0.05$), while the administration of Valproic acid concomitantly with bleomycin significantly reduced tissue levels of these

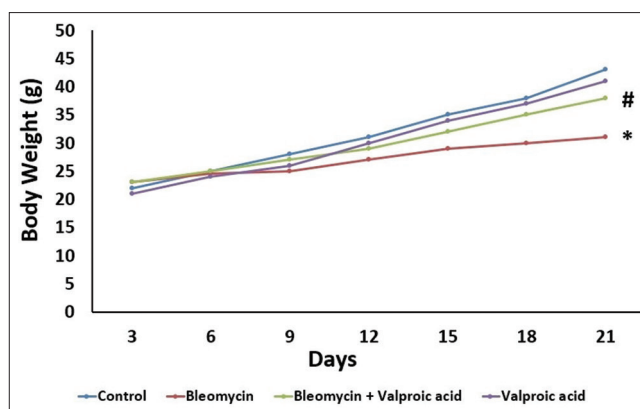


Figure 1: Effect of bleomycin and/or valproic acid on mice body weight (mean $\pm$ standard error of the mean). *Significant compared to the control group ($P < 0.05$); #Significant compared to bleomycin group ($P < 0.05$)

parameters compared to bleomycin group ($P < 0.05$). Valproic acid alone induced nonsignificant effect on the above-mentioned parameters compared to the control group ($P > 0.05$) [Table 1].

Effect of bleomycin and Valproic acid on mammalian target of rapamycin

Bleomycin induced significant increase in the tissue levels of mTOR compared to the control group ($P < 0.01$), while administration of valproic acid concomitantly with bleomycin resulted in significant decrease in the tissue levels of mTOR compared to mice treated with bleomycin alone ($P < 0.01$). Valproic acid alone induced nonsignificant effect on tissue mTOR compared to the control group ($P > 0.05$) [Figure 2].

Histopathological results

The control group showed normal appearance and size of the dermis and white adipose tissue [Figure 3a and b]. Bleomycin induced significant increase in the thickness of the dermal layer with attrition of the white dermal adipose tissue and mild inflammatory cellular infiltration [Figure 3c and d]. Administration of Valproic acid concomitantly with bleomycin resulted in the preservation of white adipose tissue layer with reduction of the dermal thickness [Figure 3e and f]. Valproic acid alone induced nonsignificant effect on the histopathological picture compared to the control group [Figure 3g and h]. By measuring the dermal thickness, significant increase in dermal thickness of bleomycin group was observed compared to the control group ($P < 0.001$), while Valproic acid taken concomitantly with bleomycin induced significant reduction of the dermal thickness compared to bleomycin group ($P < 0.001$). Administration of Valproic acid alone induced nonsignificant effect on the dermal thickness compared to the control group ($P > 0.05$) [Figure 4].

Immunohistochemical expression of α -SMA in the skin tissues

Administration of bleomycin resulted in significant increase in the positive cytoplasmic immunostaining of α -SMA in

Table 1: Effect of bleomycin and/or valproic acid on skin tissue malondialdehyde, glutathione-S-transferase, reduced glutathione, tumor necrosis factor alpha, interleukin-15 and transforming growth factor beta 1 (mean±standard error of mean)

Parameters	Control (n=10)	Bleomycin (n=8)	Bleomycin + valproic acid (n=10)	Valproic acid (n=9)
Tissue MDA (nmol/g tissue)	23.05±3.11	47.84±4.82*	28.58±2.96 [#]	24.11±3.64
Tissue GST (U/mg protein)	34.25±2.65	19.41±1.72*	27.57±2.12 [#]	33.4±2.54
Tissue GSH (mg/g tissue)	1.76±0.16	0.92±0.11*	1.53±0.15 [#]	1.88±0.18
Tissue TNF-α (pg/mg protein)	37.53±2.1	449.24±32.7*	210.6±19.21 [#]	43.25±3.74
Tissue IL-15 (pg/mg protein)	146.3±9.42	719.45±56.6*	329.12±28.7 [#]	139.51±8.93
Tissue TGF-β1 (pg/mg protein)	21.45±1.61	84.16±7.56*	43.78±3.06 [#]	23.12±1.87

*Significant compared to the control group ($P<0.05$), [#]Significant compared to bleomycin group ($P<0.05$). MDA=Malondialdehyde, GST=Glutathione-S-transferase, GSH=Glutathione, TNF=Tumor necrosis factor, IL=Interleukin, TGF=Transforming growth factor

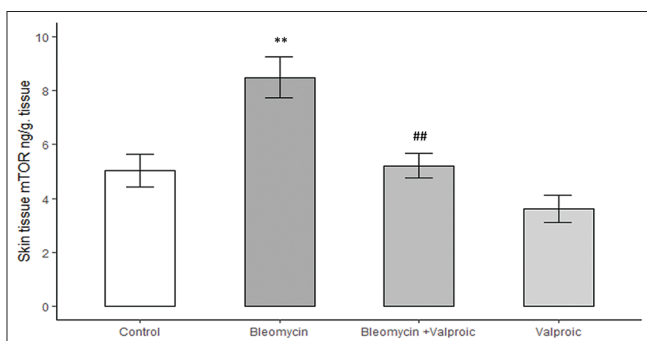


Figure 2: Effect of bleomycin and/or valproic acid on the skin tissue levels of mammalian target of rapamycin (mean ± standard error of the mean). **Mean $P < 0.01$ compared to the control group, ##Means $P < 0.01$ compared to bleomycin group

the dermal and hypodermal layers of the skin compared to the control group [Figure 5a and b]. Prophylactic use of valproic acid resulted in significant reduction in the degree of α -SMA immunostaining compared to mice treated with bleomycin alone [Figure 5c]. Administration of Valproic acid alone induced nonsignificant effect on the degree of α -SMA immunostaining compared to the control group [Figure 5d]. Quantitative analysis of the percentage of positive immunostaining showed that bleomycin significantly increased the percentage of α -SMA immunostaining compared to the control group ($P < 0.001$), whereas Valproic acid taken concomitantly with bleomycin showed significant reduction of the percentage of positive immunostaining of α -SMA compared to bleomycin group ($P < 0.001$). Administration of Valproic acid alone induced nonsignificant effect on the percentage of positive immunostaining of α -SMA compared to the control group ($P > 0.05$) [Figure 5e].

DISCUSSION

Scleroderma is a systemic disorder that affects multiple organs in the body. It is characterized by early vascular injury followed by immunological stimulation with subsequent tissue fibrosis in skin and different organs that leads to increased mortality rate due to organ dysfunction.^[15] The pathogenesis of scleroderma is complex and incompletely understood. Accordingly, no efficacious treatment is available, and most therapeutic options

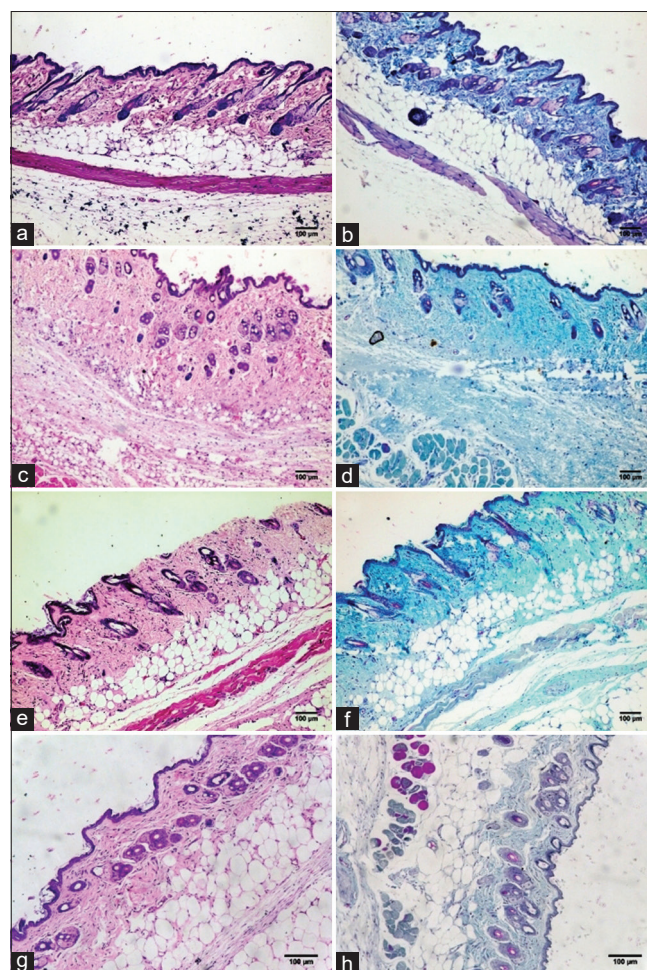


Figure 3: Sections from the skin (H and E and MT, $\times 200$) of (a and b) control group with normal appearance and size of the dermis and white adipose tissue; (c and d) Bleomycin group showing marked increase in dermal thickness with loss of white adipose tissue layer, and mild inflammatory cellular infiltrate; (e and f) Bleomycin + valproic acid group showing great reduction in dermal thickness with appearance of white adipose tissue layer, and no inflammatory infiltrate; (g and h) Valproic acid group showing normal appearance and size of dermis and white adipose tissue layer

are symptomatic and have a wide range of adverse effects.^[16] The purpose of this study was to investigate valproic acid, the well-known antiepileptic drug, for its potential ameliorative

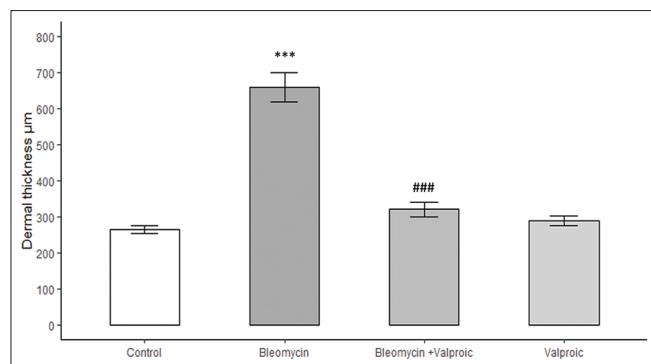


Figure 4: Effect of bleomycin and/or valproic acid on the dermal thickness of the skin (mean $\pm$ standard error of the mean). ***Mean $P < 0.001$ compared to the control group, ###mean $P < 0.001$ compared to bleomycin group

effect on skin fibrosis induced by bleomycin and to evaluate the possible mechanisms that explain its protective role with special emphasis on its effect on oxidative stress, profibrotic cytokine TGF- β 1, and the mTOR kinase activity.

In the current study, subcutaneous injection of bleomycin in the mice back on alternate days for 3 weeks resulted in reduction of body weight, especially toward the end of the study which may be due to systemic involvement of the lung with the fibrotic process as this is documented with this model.^[10] Consequently, lung fibrosis increases energy expenditure and muscle wasting with subsequent decrease of the body weight.^[17]

There is a strong evidence that oxidative stress plays an important role in the pathogenesis of scleroderma. Excessive production of reactive oxygen species (ROS) may cause endothelial dysfunction and creates proinflammatory and profibrotic status that leads to further damage and generation of the proinflammatory cytokines and growth factors.^[18] It was reported that ROS role in scleroderma involves the production of skin changes typical of the disease due to enhancement of fibroblast activation and collagen gene expression together with increased differentiation of fibroblasts to myofibroblasts through release of TGF- β 1 from activated cells.^[19] In the present study, bleomycin injection resulted in significant increase in tissue MDA levels which is a marker of lipid peroxidation, and significant reduction of skin tissue GSH levels which is a source of free thiol groups that are active component of the antioxidant capacity of the tissues denoting the presence of oxidative stress in skin tissue which is a well-known key mechanism of fibrosis induced by bleomycin as previously stated.^[18]

TGF- β 1 is an important and central pro-fibrotic cytokine that is involved in fibrogenesis in many diseases including scleroderma. Its malfunction can disrupt tissue hemostasis due to its involvement in cell proliferation, differentiation, apoptosis, and tissue regeneration.^[20] It promotes extracellular matrix synthesis, reduces collagenases produced by fibroblasts, increases generation of ROS, and promotes epithelial transformation to fibroblasts and myofibroblasts.^[21] In the

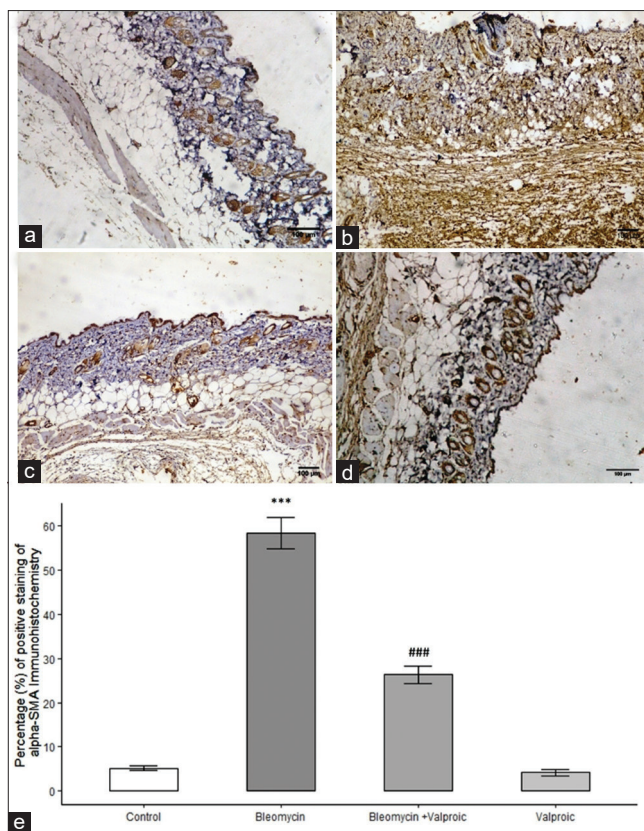


Figure 5: Immunohistochemical staining of α -smooth muscle actin ($\times 200$) in the skin tissues of (a) The control group showing minimal positive staining for α smooth muscle actin; (b) Bleomycin group showing diffuse positive staining for α smooth muscle actin which is marked in the hypdermal layer; (c) Bleomycin + valproic acid group showing mild positive staining for α smooth muscle actin; (d) Valproic acid group revealing minimal positive staining for α smooth muscle actin; (e) Percentage of α -smooth muscle actin positive immunostaining. Data are expressed as mean $\pm$ standard error of the mean. ***Means $P < 0.001$ compared to control group, ###means $P < 0.001$ compared to bleomycin group

current study, bleomycin caused significant increase in skin tissue TGF- β 1 indicating its crucial role in fibrosis as previously reported.^[22]

In the present work, subcutaneous injection of bleomycin resulted in significant elevation of skin tissue mTOR kinase level compared to the control group. mTOR is a serine/threonine kinase that plays a crucial role in the regulation of protein and lipid biosynthesis, cell cycle progression, proliferation, and survival. Recently, mTOR was reported to be implicated in collagen production, mediation of myofibroblast differentiation through TGF- β 1 signaling, and promotion of fibroblasts proliferation through inhibition of autophagy.^[23]

Oxidative stress, TGF- β 1, and mTOR elevation in bleomycin group of the present study were associated with increased dermal thickness, loss of dermal white adipose tissue layer, and mild inflammatory infiltration. In addition, fibrosis was evaluated using MT stain of collagen fibers which was evident in bleomycin group. These changes are characteristic

of human as well as the mouse models of scleroderma.^[24] Moreover, bleomycin injection resulted in significant increase in the expression of alpha SMA in skin tissue compared to the control group. Alpha SMA is a marker of differentiation of fibroblasts to myofibroblasts that are involved in active tissue fibrogenesis. Their persistence in the tissues leads to impaired repair mechanisms and increased production of the inflammatory mediators and growth factors with subsequent fibrosis.^[24]

In the current study, simultaneous administration of valproic acid with bleomycin resulted in amelioration of skin fibrosis in the mouse model manifested by reduced dermal thickness and prevention of loss of the white adipose tissue layer. This protection may be attributed to attenuation of oxidative stress as demonstrated in the present study by reduction of tissue MDA and elevation of tissue GSH and GST levels compared to mice treated with bleomycin alone. This was in agreement with Beltrán-Sarmiento *et al.*^[25] who found that monotherapy with valproic acid has antioxidant effects in epileptic children. Moreover, valproic acid in our study decreased skin tissue levels of TGF- β 1 which is the most important profibrotic cytokine and this was in the same line with previous studies investigating antifibrotic effects of valproic acid in other tissues such as the lung and the peritoneum.^[9] This reduction in TGF- β 1 levels was associated with reduced fibrosis in skin as it was reported that valproic acid can modulate Smad, the downstream effectors of serine-threonine kinase receptors activated by binding to the TGF- β 1 with subsequent reduction of collagen synthesis.^[26] On the contrary, valproic acid may increase collagen synthesis and help in cutaneous wound healing, but this was noted in the epithelial layers of the skin.^[27]

Recently, mTOR represents an attractive therapeutic target for amelioration of the fibrotic diseases.^[28] Testing inhibitors of mTOR showed a promising antifibrotic effect due to reduction of collagen synthesis induced by TGF- β 1.^[6] In the present study, prophylactic use of valproic acid significantly reduced mTOR in the skin tissue associated with significant reduction of TGF- β 1, dermal thickness and fibrosis compared to the group that received bleomycin alone. This was in accordance to a recent study showing that valproic acid can induce autophagy in prostate cancer cells through suppression of Akt/mTOR pathway.^[29] This may explain the mechanism of protection produced by valproic acid in skin fibrosis model of the current study. In addition, valproic acid had the ability to reduce alpha SMA expression, the marker of myofibroblasts that plays the major role in fibrous tissue production.

Another mechanism of protection mediated by valproic acid in the present study may be due to inhibition of histone deacetylase.^[8] Recent studies reported that histone deacetylase may have a strong association with fibrosis and vascular dysfunction in the pathogenesis of scleroderma. This was attributed to the fact that histone deacetylase is usually overexpressed in the endothelial cells and has the ability to impair angiogenesis.^[9]

CONCLUSION

The current study showed for the first time the antifibrotic effect of valproic acid in a mouse model of scleroderma induced by bleomycin which may be attributed to its antioxidant effect, reduction of the profibrotic cytokine TGF- β 1 and its suppressive effects on mTOR kinase. Further studies are needed to explore the possible clinical implication of these findings for amelioration of bleomycin-induced skin fibrosis.

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Conflicts of interest

There are no conflicts of interest.

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