

# Genistein protects dermal fibrosis in bleomycin-induced experimental scleroderma

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## Abstract

**Objective:** Genistein, a phytoestrogen, has anti-oxidant, anti-inflammatory, and anti-angiogenic properties. The aim of the present study is to evaluate the protective effect of genistein in bleomycin (BLM)-induced dermal fibrosis.

**Material and Methods:** This study involved four groups of Balb/c mice (n=10 per group). Mice in three groups were administered BLM [100 µg/day in 100 µL phosphate-buffered saline (PBS)] subcutaneously for 4 weeks; the remaining (control) group received only 100 µL/day of PBS subcutaneously. PBS or BLM was injected into the shaved upper back. Two of the BLM-treated groups also received genistein (1 or 3 mg/kg/day, subcutaneously, to the dorsal front of neck). At the end of the fourth week, all mice were sacrificed and blood and tissue samples were obtained.

**Results:** The BLM applications increased the dermal thicknesses, tissue hydroxyproline contents,  $\alpha$ -smooth muscle actin-positive cell counts, and led to histopathologically prominent dermal fibrosis. The genistein treatments decreased the tissue hydroxyproline contents and dermal thicknesses, in the BLM-injected mice.

**Conclusion:** Genistein has antifibrotic potential in BLM-induced dermal fibrosis model. However, its therapeutic potentials on human scleroderma require evaluation in future studies.

**Keywords:** Scleroderma, dermal fibrosis, genistein

## Introduction

Scleroderma is a chronic inflammatory disease characterized by widespread fibrosis of the skin and internal organs (1-3). Vasculopathy and immune activations are the main factors in the pathogenesis of scleroderma, although its pathogenesis is not yet fully understood (1-4). Vasculopathy, characterized by intimal fibroproliferation and episodic vasospasms, leads to endothelial injury and subsequently increases adhesions and migrations of inflammatory cells (4). Moreover, intimal fibroproliferation leads to severe and chronic hypoxia (5). Hypoxia is a major stimulus for angiogenesis, leading to the expression of pro-angiogenic molecules, particularly for vascular endothelial growth factor (VEGF), which is a well-characterized regulator of physiological and pathological angiogenesis (6, 7).

Activated endothelial and inflammatory cells activate the fibroblastic cells through cell-cell interactions or by cytokines and growth factors, including interleukin (IL)-4 and transforming growth factor (TGF)- $\beta$ 1 (1-3). Activated fibroblasts (myofibroblasts) are the key effectors of extracellular matrix (ECM) production and they also express profibrotic cytokines and growth factors, including IL-6, TGF- $\beta$ 1, platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF) (1-3). Therefore, activated fibroblasts exhibit autocrine behavior, and they do not need other exogenous stimuli for the persistence of their activation, in scleroderma (1-3). In addition to the autonomy of fibroblasts, the transformation of non-fibroblastic cells to fibroblastic cells is another possible pathogenic mechanism in scleroderma. Endothelial cells cultured with fibroblast growth factor (8) and exposed to tumor necrosis factor alpha (TNF- $\alpha$ ) or IL-1 $\beta$  (9) have been observed to transform into myofibroblastic cells in in vitro experimental studies. Endothelial-myofibroblastic cell transformation by the application of homocysteine, a potent oxidant, has also been reported (10).

Genistein, an isoflavonoid present in soy products (11), has anti-oxidant, anti-proliferative, and anti-angiogenic properties (12). Anti-angiogenic effect of genistein is associated with the suppression of VEGF and VEGF receptor (VEGFR)-1 (fms-like tyrosine kinase-1) expressions (13), inhibition of tyrosine kinase activity (12), and reduction of proteases [urokinase-type plasminogen activator (uPA) and matrix metalloproteinase (MMP)-1] levels that is induced by VEGF (14).



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The aim of the present study was to evaluate the possible protective effectiveness of genistein in bleomycin (BLM)-induced dermal fibrosis in an experimental scleroderma model.

## Material and Methods

### Animals and experimental protocols

Forty specifically pathogen-free Balb/c mice (6 weeks old, female), weighing 20–25 g, were used for the experimental procedures. They were randomly classified into four groups (n=10 in each group). Mice were housed in the animal facility of Firat University Experimental Research Center, maintained in a climate-controlled environment with a 12 h light/dark cycle in polystyrene cages containing wood shavings, and fed standard rodent chow and water, ad libitum. The Animal Care and Ethics committee of Firat University approved the care of mice and the experimental procedures.

Mice in the control group received 100 µL/day of phosphate-buffered saline (PBS) everyday to the shaved upper back skin, subcutaneously, for 4 weeks. To induce dermal fibrosis, the remaining three groups received 100 µg BLM (Bleocin; Nippon Kayaku, Tokyo, Japan) dissolved in 100 µL PBS and sterilized by filtration (0.2 µm filter) to the shaved upper back skin for 4 weeks. Two groups of these BLM-treated mice also received subcutaneous 1 or 3 mg/kg/day of genistein (Sigma; İstanbul, Turkey) dissolved in dimethyl sulfoxide, as previously described (15). Genistein was injected to the dorsal front of neck daily for 4 weeks.

All animals were sacrificed by cervical dislocation under anesthesia with ketamine hydrochloride, on the day following the final applications at the end of the fourth week. The blood samples and upper back skins were harvested for further examination.

### Enzyme-linked immunosorbent assay (ELISA) of serum cytokines

Blood samples were extracted by cardiac puncture, and sera were obtained after centrifugation at 3000 rpm for 10 min and stored at –20°C, until the day of the analysis. Serum IL-2, IL-4, and TGF-β<sub>1</sub> levels were measured using appropriate commercial kits (Biosource International; Camarillo, California, USA) by the ELISA method.

### Histopathology and Immunohistochemistry

The skin specimens were divided in two parts: one was fixed with 10% formalin solution and the other was stored immediately at –80°C for tissue hydroxyproline (OH-proline) content assay.

**Table 1.** Cytokine level, tissue OH-proline contents, and histopathological findings in the study groups

|                              | PBS        | BLM                     | GEN (1 mg/kg)           | GEN (3 mg/kg)           |
|------------------------------|------------|-------------------------|-------------------------|-------------------------|
| IL-2 (pg/mL)                 | 7.53±0.45  | 8.44±2.82               | 7.25±0.27 <sup>†</sup>  | 6.81±0.38 <sup>†</sup>  |
| IL-4 (pg/mL)                 | 7.04±0.13  | 8.43±2.90               | 7.30±0.42               | 7.39±0.78               |
| TGFβ1 (pg/mL)                | 1098±486   | 1363±487                | 1677±261                | 1384±427                |
| OH-proline (mg/g dry tissue) | 0.52±0.19  | 1.71±0.58 <sup>§</sup>  | 0.76±0.14 <sup>¶</sup>  | 0.95±0.28 <sup>¶</sup>  |
| Dermal thickness (µm)        | 192.8±53.5 | 435.5±77.8 <sup>§</sup> | 279.0±63.1 <sup>¶</sup> | 336.8±92.2 <sup>†</sup> |
| Inflammatory cells (/HPF)    | 7.9±3.1    | 34.4±27.5 <sup>§</sup>  | 11.2±5.1 <sup>†</sup>   | 15.6±6.5 <sup>†</sup>   |
| α-SMA+ cells (/HPF)          | 1.02±0.4   | 2.4±1.1 <sup>§</sup>    | 1.6±0.6 <sup>†</sup>    | 2.01±0.8                |

Data are expressed as mean±standard deviation.

When compared to the PBS group: <sup>†</sup>p<0.001.

When compared to the BLM group: <sup>§</sup>p<0.05, <sup>†</sup>p<0.01, <sup>¶</sup>p<0.001.

PBS: phosphate-buffered saline; BLM: bleomycin; GEN: genistein; IL: interleukin; TGF: transforming growth factor; OH-proline: hydroxyproline; α-SMA+: alpha-smooth muscle actin-positive; HPF: high power field

The skin specimens were embedded in paraffin and cut into 4-µm thick sections using a microtome. They were then stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). Dermal thickness (measured from the epidermal–dermal junction to dermal–fat junction) was determined from five randomly selected sites of two or more skin sections in each animal by ×100 magnification, using an Olympus BX-50 light microscope.

After staining with streptavidin biotin [Actin Muscle Ab-6 (MSA06); Neomarkers, Fremont, California, USA], the number of alpha-smooth muscle actin-positive (α-SMA+) fibroblastic cells (myofibroblasts) was counted using a micrometer under a light microscope, at ×400 high-power field (HPF) from five randomly selected fields in each specimen, and the mean number was calculated.

### Measurement of hydroxyproline

Collagen deposition was estimated by determining the total OH-proline content of the skin. The stored skin specimens were washed with normal saline, dried in an oven at 100°C for 72h, and hydrolyzed with 12 N hydrochloric acid at 130°C for 3h, according to Woessner's method (16). After neutralizing with sodium hydroxide, the hydrolysates were diluted with distilled water. OH-proline in the hydrolysates was calorimetrically assessed at 560 nm with p-dimethylaminobenzaldehyde, and expressed as mg/g dry tissue.

### Statistical analysis

Statistical evaluations were performed using the SPSS package program, version 11.0. Data were presented as mean±standard deviation. Statistical analyses were performed by Kruskal–Wallis one-way analysis of variance and Mann–Whit-

ney U test for dual-comparisons. P value of <0.05 was considered to be statistically significant.

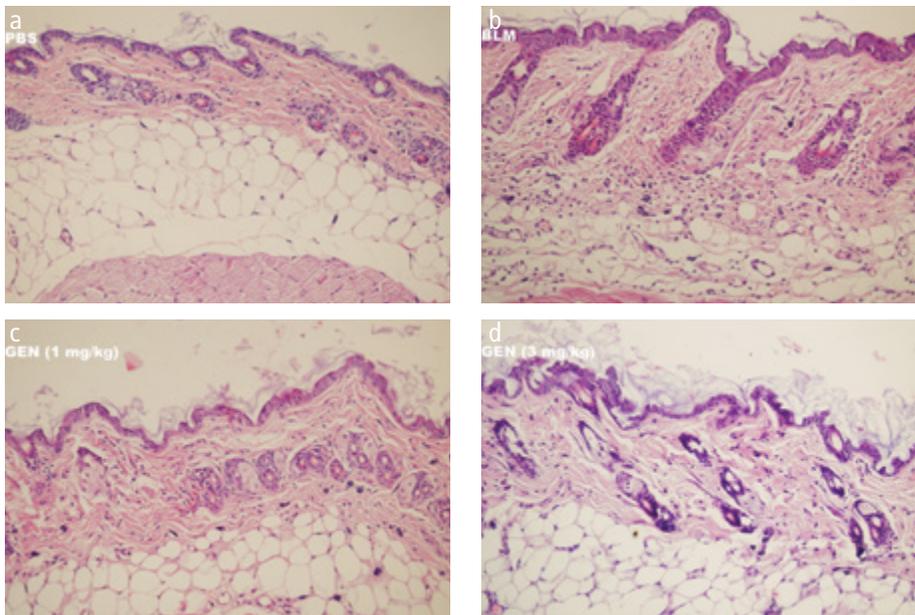
## Results

Daily BLM injections for 4 weeks caused increases in inflammatory cell infiltration, in the number of α-SMA+ fibroblastic cells, and in dermal thickness compared with the PBS-treated control group (Table 1, Figure 1a-d, 2a-d). In addition, serum TGF-β1 level was relatively higher in the BLM-treated group. The examinations with H&E and MT staining revealed an increased deposition of collagen and histologically prominent dermal fibrosis, characterized by thickened collagen bundles with cellular infiltrates that mimicked scleroderma in the BLM-treated group (Figure 1a). The decrease in the amount of subcutaneous fat tissue was also observed in BLM-treated mice (Figure 1b).

The decreases in inflammatory cell infiltration and α-SMA expressions were prominent in genistein treated group with a dosage of 1 mg/kg (Figure 2c, d). Genistein applications with all doses decreased the dermal thickness (Figure 1c, d) and OH-proline content compared to the BLM-treated sham group (Table 1). Improvements in the dermal thickness and OH-proline content were more prominent in the treatment groups as they reached greater reduction in the inflammatory cell infiltration and expression of α-SMA (Table 1).

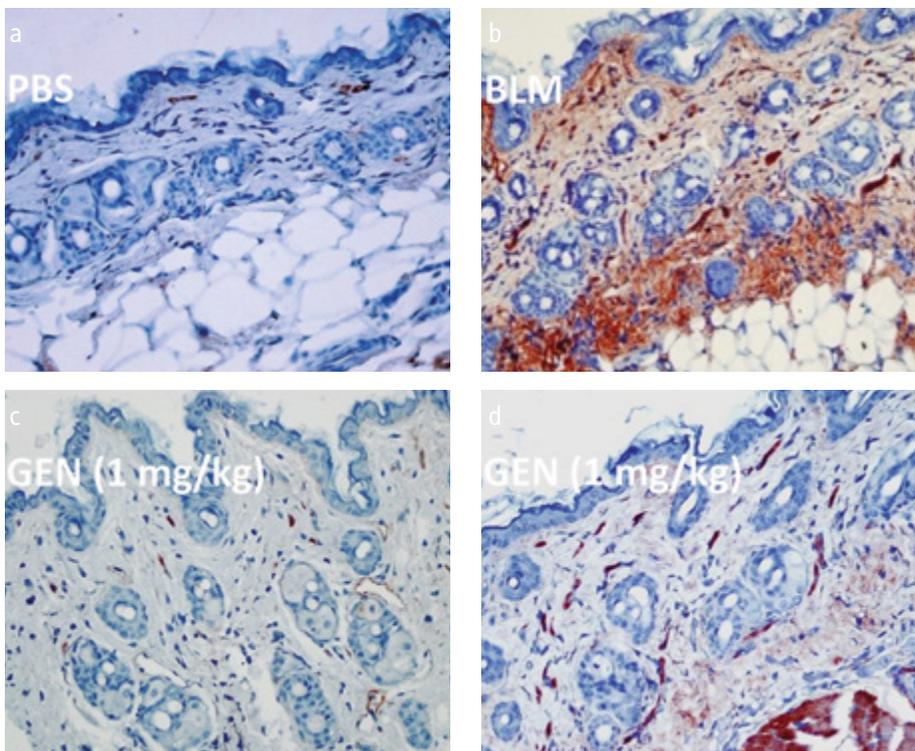
## Discussion

In this study, the preventive antifibrotic effect of genistein in the early stages of BLM-induced dermal fibrosis was investigated. Repeated BLM injections caused inflammatory cell infiltration, fibroblast activation, and dermal fibrosis. On the other hand, the application of genistein decreased BLM-induced inflam-



**Figure 1. a-d.** Representative photomicrographs of hematoxylin and eosin (H&E) staining ( $\times 100$ ), normal histopathological view in the PBS-injected control group (a), increased dermal thickness and infiltration of inflammatory cells in the BLM-injected sham group (b), genistein treatments decreased the dermal thickness in the BLM-injected mice (c, d)

PBS: phosphate-buffered saline; BLM: bleomycin; GEN: genistein



**Figure 2. a-d.** Representative photomicrographs of alpha-smooth muscle actin ( $\alpha$ -SMA) staining ( $\times 200$ ), normal expression in the PBS-injected control group (a), increased  $\alpha$ -SMA positive cell count in the BLM-injected sham group (b), genistein treatments decreased  $\alpha$ -SMA positive cell count (c, d)

PBS: phosphate-buffered saline; BLM: bleomycin; GEN: genistein

matory cell infiltration, fibroblast activation, deposition of collagen in dermal tissue, and thickening of the skin.

Genistein, a phytoestrogen and an isoflavonoid present in soy products, ameliorated BLM-in-

duced dermal fibrosis in the present study. Previous *in vivo* studies have documented that genistein prevented fibrosis of heart, kidney, and liver (17-19). Genistein is a specific inhibitor for tyrosine kinases (12) and exerts direct anti-proliferative effects on human cell lines.

Its anti-proliferative effects on fibroblasts may be one cause of its protective effect on dermal fibrosis. Moreover, it suppresses the production of adhesion molecules [intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)] from human endothelial cells (20). Therefore, genistein exerts anti-proliferative and anti-angiogenic properties (21). It also inhibits the adhesive and migratory capacity of inflammatory cells (22) and maturation of lymphocytes (23). It suppresses lipopolysaccharide (LPS) induced expressions of cytokines by inflammatory cells (11). Therefore, the anti-inflammatory action of genistein may be another cause of its protective effect on dermal fibrosis.

Genistein decreases lipid peroxidation products, such as  $F_2$ -isoprostane (24), and thiobarbituric acid reactive substances (TBARS) (25). Several studies demonstrate that excessive oxidative stress is present in scleroderma, either through a reduction of antioxidants including ascorbic acid,  $\alpha$ -tocopherol and selenium or by an increase of oxidants, including malondialdehyde,  $F_2$  isoprostanes, and reactive oxygen species (ROS) (26-31). Balbir-Gurman et al. (28) reported that treatment with a synthetic prostacyclin analogue normalizes antioxidant activity. This result indicates that damaged perfusion may be the cause of oxidative stress in scleroderma (28). On the other hand, during inflammation, there is significant production of ROS by non-phagocytic cells and cytokines promote inflammatory activation and ROS production (29). Oxidative stress leads to exacerbated inflammation by upregulating cytokines (30) and induces autoantigen fragmentation, leading to autoantibody production (31). Moreover, it may play a role in the development of the T cell repertoire in scleroderma as in other autoimmune disorders (29). Therefore, the antioxidant properties of genistein (24, 25) may be another cause of its antifibrotic action.

In addition to anti-oxidant and anti-inflammatory actions genistein exerts anti-angiogenic property which is reported to be associated with the inhibition of VEGF (13, 14). The over-expression of VEGF in serum (32, 33) and skin samples (6, 7) of patients with scleroderma has been demonstrated. In the present study, genistein ameliorated dermal fibrosis in the BLM-induced scleroderma model. This antifibrotic effort may be caused by its potential action on VEGF.

Scleroderma, a chronic inflammatory and fibrotic disease, is more frequent in women than in men. Moreover, the fibrotic potentials of estrogen have previously been reported (34, 35). It may

be surprising that genistein protects the area of fibrosis. Because genistein is a phytoestrogen (13) and while it has anti-oxidant, anti-proliferative and anti-angiogenic properties (12-14), genistein also modulates estrogen receptor  $\beta$  (ER $\beta$ ) activity. It has been demonstrated that estrogen exerts profibrotic effects via acting on ER $\alpha$ , but ER $\beta$  protects the area of fibrosis (35). Moreover, it has been reported that genistein ameliorates heart, kidney, and liver fibrosis in vivo (17-19).

This study has some limitations. Firstly, it would have been better to examine the efficacies of treatments at later stages of pre-existing fibrosis, in addition to the early stages of dermal fibrosis. Secondly, analyzing serum levels of cytokines may be another limitation, because dermal fibrosis is localized and restricted to the injected areas in the BLM-induced scleroderma model. The methods for detecting local expression of cytokines could have been more appropriate.

In conclusion, genistein has antifibrotic actions on dermal fibrosis, experimentally induced by BLM. However, therapeutic potentials of genistein in human scleroderma require to be evaluated by further studies.

**Ethics Committee Approval:** Ethics Committee approval was received for this study from The Animal Care and Ethics committee of Firat University.

**Informed Consent:** N/A

**Peer-review:** Externally peer-reviewed.

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