

Original Article

Inhibitory effect of kaempferol on skin fibrosis in systemic sclerosis by the suppression of oxidative stress

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ABSTRACT

Background: There is growing evidence that vasculopathy-induced hypoxia and oxidative stress enhance the process of fibrosis in systemic sclerosis (SSc). Kaempferol is a natural flavonoid widely found in various vegetables and fruits, and has been reported to have excellent antioxidant activity.**Objective:** Objective was to elucidate the effect of kaempferol on skin fibrosis and the mechanism of the inhibitory regulation of fibrosis by kaempferol.**Methods:** We assessed the effect of intraperitoneally administered kaempferol on bleomycin-induced dermal fibrosis in mice. The effect of kaempferol on oxidative stress in bleomycin-treated mice and SSc fibroblasts was assessed *in vivo* and *in vitro*.**Results:** We identified that kaempferol injection significantly inhibited bleomycin-induced dermal fibrosis in mice. The number of α SMA⁺ myofibroblasts, CD3⁺ T-cells, and CD68⁺ macrophages in lesional skin was significantly decreased by kaempferol injections. Kaempferol administration also significantly suppressed the bleomycin-induced oxidative stress signal in OKD48 mice. Additionally, mRNA levels of oxidative stress-associated factors, such as HO-1 and NOX2, as well as inflammatory and pro-fibrotic cytokines, including IL-6, TGF- β and TNF α in sclerotic skin were significantly decreased by kaempferol. Kaempferol also reduced bleomycin-induced TUNEL⁺ apoptotic cells in the lesional skin of bleomycin-treated mice. Furthermore, the oxidant-induced intracellular accumulation of reactive oxygen species (ROS) in SSc fibroblasts was inhibited by kaempferol treatment. In addition, the oxidant-induced apoptosis of SSc fibroblasts was decreased by kaempferol *in vitro*.**Conclusion:** Kaempferol might improve bleomycin-induced fibrosis by reducing oxidative stress, inflammation, and oxidative cellular damage. Administration of kaempferol might be an alternative treatment for skin fibrosis in SSc.

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1. Introduction

Systemic sclerosis (SSc) is a connective tissue disorder characterized by the development of fibrosis in the skin and internal organs, as well as microvascular dysfunction [1,2].

Abbreviations: ATP, adenosine triphosphate; ECs, endothelial cells; HO-1, Heme Oxygenase 1; IL-6, interleukin-6; Nox, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species; SSc, systemic sclerosis; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; Trx2, thioredoxin 2; TUNEL, terminal deoxynucleotide transferase dUTP nick end-labeling.

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Vasculopathy occurs at an early step of SSc. Almost all patients present Raynaud's phenomenon as an initial symptom, and frequently show other manifestations of vascular diseases, including ischemic digital ulcers, pulmonary arterial hypertension, and renal arterial involvement associated with malignant hypertension and renal failure. Although the pathogenesis of SSc remains unknown, it has been elucidated that vasculopathy-induced hypoxia and oxidative stress play an important role in the prognosis of SSc [3–8]. Vasculopathy in patients with SSc is characterized by the activation of endothelial cells (ECs) and altered vascular tone. These pathological changes involve the induction of pro-inflammatory cytokines and abnormal angiogenesis/vasculogenesis regulators, as well as the loss of redox regulation, leading to oxidative stress and hypoxia [3]. There are many previous reports which demonstrated that the serum levels of oxidative stress-related factors, such as nitric oxide,

malondialdehyde, asymmetric dimethylarginine, hydroperoxide, and homocysteine, increased in patients with SSc [4,5]. Anti-oxidative biomarkers, such as superoxide dismutase and vitamin C, were lower in the serum of patients with SSc compared to healthy controls [4]. In addition, mice treated with agents that induce the release of free radicals, such as hydroxyl radicals and peroxy-nitrites, exhibited cutaneous and lung fibrosis [6]. Furthermore, it has been reported that edaravone, a free radical scavenger, has a significant inhibitory effect on fibrosis both in the bleomycin-induced SSc mouse model and in tight skin mice [7]. Selective nicotinamide adenine dinucleotide phosphate (NADPH) oxidases 4 (NOX4) inhibitor exerted a highly effective anti-fibrotic effect in various animal models of tissue fibrosis [8]. These pieces of evidence indicate that vasculopathy-induced hypoxia and oxidative stress might promote fibrosis in SSc.

Flavonoids are polyphenol compounds mainly contained in plants and play an important role in our health. Kaempferol (3,4',5,7-tetrahydroxyflavone) is a natural flavonoid abundantly found in tea, broccoli, apples, strawberries, and beans [9]. A number of studies have shown that kaempferol has a wide range of beneficial pharmacological activities, including excellent anti-oxidation [10–13], anti-inflammation [14], and anti-tumor growth [15]. However, the effect of kaempferol on the skin fibrosis in mice model *in vivo* and the mechanisms of the inhibitory effect for fibrosis by kaempferol are unclear. Therefore, in this study, we examined the effect of kaempferol on skin fibrosis in bleomycin-induced fibrosis mice model *in vivo* and the mechanisms of the inhibitory effects of oxidative stress and inflammation by kaempferol *in vivo* and *in vitro*.

2. Materials and methods

2.1. Animals

All experiments were approved by the Gunma University Animal Care and Experimentation Committee (#17-047, #15-053), and carried out in accordance with the approved guidelines. C57BL/6 mice were purchased from the SLC (Shizuoka, Japan). Eight- to 12-week-old mice were used for all experiments. The mice were maintained in the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

2.2. Bleomycin-induced skin fibrosis model

Dermal fibrosis was induced in 8-week-old C57BL/6 or OKD48 (Keap1-dependent oxidative stress detector, NO-48) mice with injections of bleomycin. Injections of 300 μ l of bleomycin (Nippon Kayaku) at a concentration of 1 mg/ml were given 5 times per week for 2 weeks as previously described [16,17]. To examine the effect of kaempferol, mice received intraperitoneally kaempferol (40 mg/kg/day) dissolved in 100 μ l of DMSO or DMSO alone 5 times per week for 2 weeks.

2.3. Quantitative assessment of collagen content

Total soluble collagen in the skin was quantified using a Sircol collagen assay (Biocolor, County Antrim, UK) according to the manufacturer's protocol and the previously described protocols [17,18].

2.4. Western blotting

Western blot analyses were performed according to the previously described protocols [16,17]. After washing with ice-cold

PBS, the skin samples were disrupted in a RIPA buffer. Lysates were centrifuged, and the resulting supernatants were subjected to SDS-PAGE, followed by immunoblot analysis using anti-connective tissue growth factor (CTGF) Ab (Santa cruz), anti-heme oxygenase 1 (HO-1) Ab (Abcam), anti-active caspase-3 Ab (Abcam) and anti-GAPDH Ab (Santa Cruz). HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were used with ECL prime (GE healthcare) to image immunoblots.

2.5. Histological examination

Sections (4 μ m thick) of mouse skin in paraffin were stained with hematoxylin and eosin (H&E) or Masson's trichrome. Skin fibrosis was quantified by measuring the thickness of the dermis, which was defined as the distance from the epidermal-dermal junction to the dermal-subcutaneous junction, at 6 randomly selected microscopic fields. For immunohistochemical staining, tissue sections of mouse skin were treated for antigen retrieval with a pressure cooker. After blocking, the sections were incubated with anti- α SMA antibody (Sigma-Aldrich, St. Louis, MO), anti-CD3 antibody (Abcam, Cambridge, UK) and anti-CD68 antibody (Bio-Rad, Hercules, CA). After washing, the sections were incubated with a horseradish peroxidase-labeled polymer-conjugated secondary antibody (EnVision⁺; Dako). Finally, color was developed with 3,3'-diaminobenzidine tetrahydrochloride. Numbers of α SMA⁺, CD3⁺ and CD68⁺ cells in the dermis were determined by counting in six random microscopic fields in 4–6 mice per group.

2.6. RNA isolation and quantitative reverse transcription-polymerase chain reaction

To analyze the mRNA expression levels in the bleomycin-induced fibrotic skin by using RT-PCR, whole skin samples from the bleomycin injected site were used. Total RNA was isolated using RNeasy Mini Kits (Qiagen, Valencia, CA) and was subjected to reverse transcription with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR was performed with the SYBR system (Applied Biosystems, Foster City, CA) by using ABI 7300 real-time PCR instrumentation. SYBR probes and primers were purchased from Sigma and Takara Bio Inc. (Otsu, Japan). Normalization and fold changes were calculated using the comparative Ct method.

2.7. Detection of luminescent signals

Detection of luminescent signals in OKD48 mice was performed as described previously [19–21]. Mice were sacrificed and the skin was surgically removed and immersed in 0.3 mg/mL VivoGloTM Luciferin, *in vivo* Grade (Promega, Tokyo, Japan) dissolved with PBS. As soon as possible, the collected skin was placed in the imaging chamber of the *in vivo* imaging system (IVIS; Perkin Elmer, Waltham, MA). Data were collected with high-sensitivity/10-min exposure and analyzed using Living Image software (Xenogen).

2.8. Apoptosis assay

The presence of apoptotic cells in the skin sections were assessed using a terminal deoxynucleotide transferase dUTP nick end-labeling (TUNEL) staining kit (Roche Diagnostics, Indianapolis, IN) as described previously [20–23]. Images (six fields per section) were taken and visualized with a FV10i-DOC confocal laser scanning microscope (Olympus). The number of apoptotic cells

was determined by counting TUNEL and DAPI double-positive nuclei in the field, as previously reported [20–23].

2.9. ROS detection assay in vitro

Human dermal fibroblasts were obtained by skin biopsies of affected dorsal forearm areas from 3 diffuse cutaneous type of SSc patients, according to the classification by LeRoy et al., [24] and age, race and gender matched healthy volunteers. The study was approved by the local research ethics committee of Gunma University. This study was conducted according to the Declaration of Helsinki principles. Cells were stimulated with 1 mM H₂O₂ with or without kaempferol for 2 h, and then the reactive oxygen species (ROS) levels were measured with the DCFDA Cellular ROS Detection Assay Kit (Abcam) according to the manufacturer's protocol and as previously reported [20,21,25].

2.10. Apoptosis and necrosis analysis with flow cytometry

Flow cytometric analysis of apoptosis was performed as described previously [20,21,25]. SSc fibroblasts were incubated in control medium or kaempferol with or without H₂O₂ (0.5 mM) for 12 h. Cells were treated with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Bioscience) and 7-amino-actinomycinD (7-AAD), and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Cells that stained positive for Annexin V and negative for 7-AAD were considered to be apoptotic cells and stained double positive for Annexin V and 7-AAD were considered to be necrotic cells.

2.11. Statistical analysis

P values were calculated using one-way analysis of variance, followed by Bonferroni's post test. Error bars represent standard errors of the mean, and the numbers of experiments (*n*) are as indicated.

3. Results

3.1. Kaempferol suppressed the bleomycin-induced dermal fibrosis in mice

First, to assess the effect of kaempferol on skin fibrosis *in vivo*, we compared the bleomycin-induced skin fibrosis treated with or without kaempferol. Bleomycin-enhanced dermal thickness was significantly suppressed by kaempferol injections (Fig. 1A, B). Dermal thickness in mice without bleomycin treatment was not changed by kaempferol injections (data not shown). We confirmed that the amount of collagen in the skin revealed by Masson's trichrome staining was enhanced by bleomycin, and this enhancement was inhibited by kaempferol injections (Fig. 1C). In addition, the increase in the amount of collagen in the skin induced by bleomycin treatment was also significantly inhibited by kaempferol treatment (Fig. 1D). Connective tissue growth factor (CTGF) is the member of matricellular proteins associated with fibrosis. CTGF is overexpressed in SSc fibroblasts and plays an important role in the production and maintenance of fibrotic lesions in SSc [26]. We found that bleomycin-induced CTGF expression was also inhibited by kaempferol treatment (Fig. 1E). The number of αSMA⁺ myofibroblasts in lesional skin in bleomycin-treated mice was increased, and these cells were significantly decreased by kaempferol injections (Fig. 1F). These results suggested that kaempferol might have the potential to suppress skin fibrosis induced by bleomycin injection *in vivo*.

3.2. Kaempferol injection reduced oxidative stress in the bleomycin-induced fibrotic skin in vivo

Since the excellent anti-oxidant activity of kaempferol is well known [10–13], we next examined the effect of kaempferol on oxidative stress in the skin using the bleomycin-induced fibrosis mouse model. OKD48 mice have a transgene encoding a modified Nrf2, which is an essential transcription factor for the expression of anti-oxidative stress genes [19,27]. By using this mouse strain, oxidative stress *in vivo* could be detected with luminescence signals [19–21]. After injection of bleomycin for 5 days, strong luminescence signal was detected in the bleomycin-induced fibrotic skin, and this signal was markedly reduced by kaempferol injection (Fig. 2A, B). Additionally, mRNA expression levels of oxidative stress-associated factors, including HO-1, NADPH oxidase 2 (NOX2), and thioredoxin 2 (Trx2) in the bleomycin-induced fibrotic skin were examined using real-time PCR. HO-1 is an important enzyme with antioxidant effect [28]. Biological function of NOX enzymes is the generation of reactive oxygen species (ROS) [29]. Trx2 is a multi-functional protein which is essential for protection against oxidant-induced apoptosis [30]. We found that mRNA expressions of HO-1 and NOX2 were significantly enhanced by bleomycin treatment, and bleomycin-induced mRNA expressions of HO-1 and NOX2 were significantly inhibited by the administration of kaempferol (Fig. 2C). Bleomycin-induced protein expression of HO-1 was also significantly inhibited by kaempferol treatment (Fig. 2D). However, Trx2 mRNA levels in the skin treated with bleomycin were not changed by kaempferol treatment. These results suggested that bleomycin-induced oxidative stress in the skin might be inhibited by kaempferol injection.

3.3. Kaempferol suppressed the number of inflammatory cells and the inflammatory and pro-fibrotic cytokines in the skin of bleomycin-treated mice in vivo

It has been reported that bleomycin-treated mice also mimic the inflammatory component of patients with SSc [31]. Since kaempferol has been reported to bring benefits of attenuation of inflammation [14,32], we next investigated the effect of kaempferol on bleomycin-induced inflammatory cells and cytokines in mice. After injection of bleomycin for 5 days, the number of CD3⁺ T cells and CD68⁺ macrophages in the dermis of bleomycin-treated mice was significantly higher than those in the non-treated mice, and kaempferol treatment significantly reduced the number of these cells (Fig. 3A). Additionally, the mRNA expression levels of inflammatory and pro-fibrotic cytokines, including interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β), in the lesional skin of bleomycin-treated mice were enhanced, and these levels were significantly reduced by kaempferol treatment (Fig. 3B). These results suggested that kaempferol might suppress the infiltration of inflammatory cells and the inflammatory and pro-fibrotic cytokines in the lesional skin of bleomycin-treated mice *in vivo*.

3.4. Kaempferol reduced bleomycin-induced apoptosis in vivo and inhibited the oxidant-induced intracellular accumulation of ROS and apoptosis in SSc fibroblasts in vitro

We next examined the effect of kaempferol on the number of apoptotic cells in the lesional skin of bleomycin-treated mice. After injection of bleomycin for 5 days, the increase in the number of TUNEL⁺ apoptotic cells in the bleomycin-injected site was significantly decreased by kaempferol injection (Fig. 4A). We also examined caspase-3 activity, which is the apoptosis-related marker. Bleomycin-induced caspase-3 activity in the lesional skin was significantly inhibited by kaempferol treatment *in vivo*.

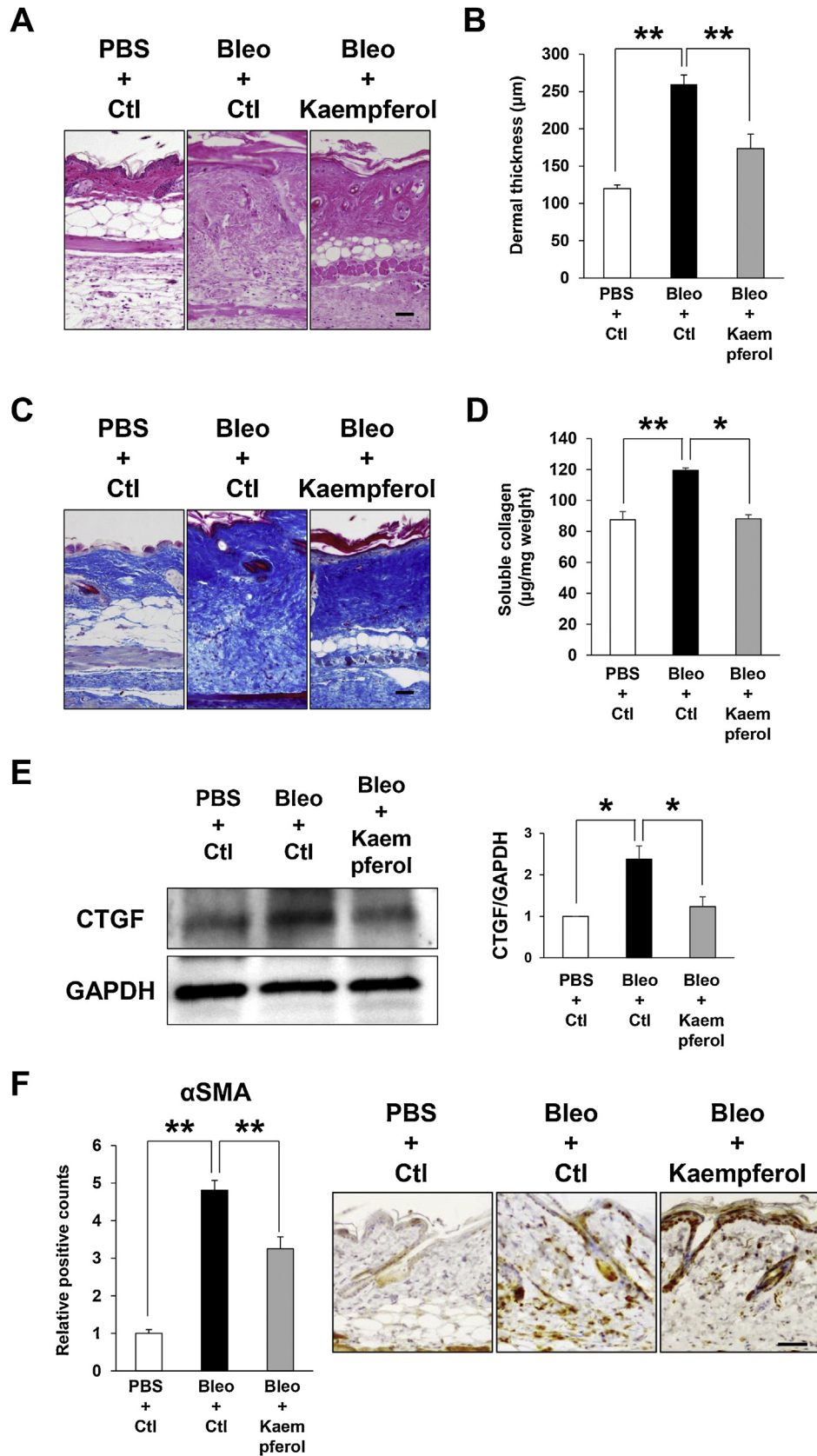


Fig. 1. Kaempferol suppressed the bleomycin-induced dermal fibrosis in mice. (A, C) Representative images of H&E staining (A) or Masson-Trichrome staining (C) of the skin in mice treated with subcutaneous injections of control PBS or bleomycin, and treated with intraperitoneal injection of kaempferol or control DMSO. Scale bar = 50 μm. (B) Quantification of dermal thickness of the lesional skin in mice. Values were determined in three random microscopic fields in $n = 5-6$ mice per groups. (D) The amount of soluble collagen amount in the skin tissue treated with control PBS or bleomycin, and treated with intraperitoneal injection of kaempferol or control DMSO. $n = 3$ mice per

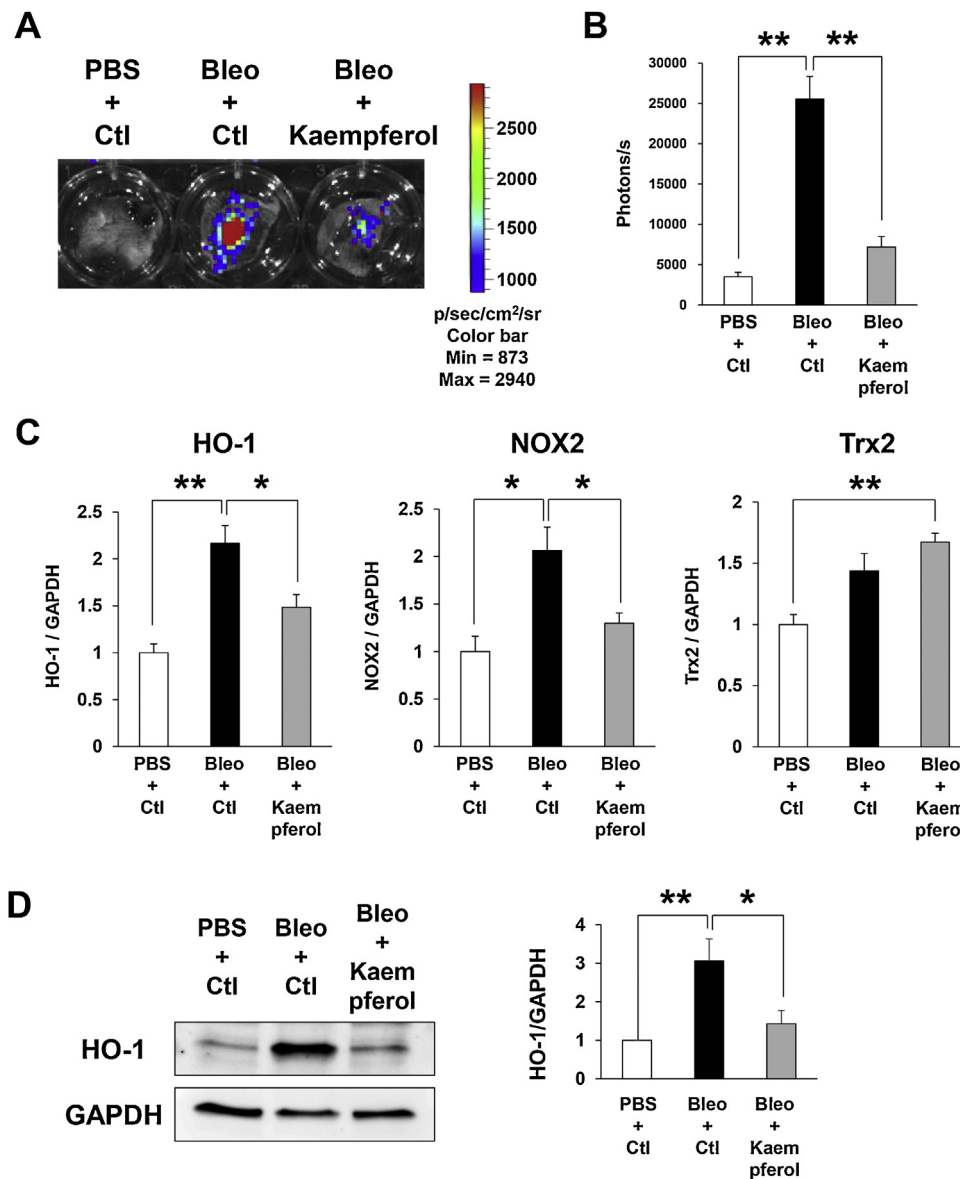


Fig. 2. Kaempferol injection reduced oxidative stress in the bleomycin-induced fibrotic skin *in vivo*. (A) Representative image of luminescence signals in the skin of OKD48 mice treated with subcutaneous injections of control PBS or bleomycin, and treated with intraperitoneal injection of kaempferol or control DMSO for 5 days. The color scale bar shows the photon counts (photon (p)/sec/cm²/sr). (B) Quantification of luminescence signals in bleomycin or PBS-injected skin site in OKD48 mice. $n = 3-5$ in each group. (C) mRNA levels of oxidative stress-associated factors, HO-1, NOX2 and Trx2 in the skin treated with subcutaneous injections of bleomycin of control PBS, and treated with intraperitoneal injection of kaempferol or control DMSO for 5 days. $n = 4-8$ mice in each group. (D) Protein levels of HO-1 in the skin tissue treated with control PBS or bleomycin, and treated with intraperitoneal injection of kaempferol or control DMSO by immunoblotting. $n = 5$ mice per groups. Quantification of relative levels of HO-1 were accomplished *via* densitometry using ImageJ. mRNA and protein levels in control mice were assigned values of 1. Values represent mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.05$.

(Fig. 4B). It has been reported that ROS is produced from SSc fibroblasts by oxidative stress, and ROS can cause several abnormalities, such as ECs damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines, including PDGF and TGF- β [33]. Therefore, we next investigated the effect of kaempferol on H_2O_2 -induced intracellular ROS accumulation in SSc fibroblasts. H_2O_2 -induced intracellular ROS accumulation was suppressed by kaempferol treatment (Fig. 4C). Next, the effect of kaempferol on H_2O_2 -induced apoptosis in SSc fibroblasts was examined using flow cytometry. The number of apoptotic cells

(Annexin V⁺ and 7-AAD⁻) was increased by H_2O_2 treatment (Fig. 4D). On the other hand, administration of kaempferol significantly reduced these cells in a dose-dependent manner (Fig. 4D). Spadoni et al. reported that the expression levels of NOX2 increased in SSc skin fibroblasts compared to normal skin fibroblasts [34]. Therefore, we next examined the mRNA expressions of NOX2 in the normal and SSc fibroblasts treated with or without kaempferol. Similar to previous results, we identified that the expression of NOX2 was increased in SSc fibroblasts compared to normal skin fibroblasts (Fig. 4E). In addition, we found that the increased expression of NOX2 in SSc fibroblasts was significantly

groups. (E) Protein levels of CTGF in the skin tissue treated with control PBS or bleomycin, and treated with intraperitoneal injection of kaempferol or control DMSO by immunoblotting. $n = 3$ mice per groups. Quantification of relative levels of CTGF were accomplished *via* densitometry using ImageJ. The expression level in control mice was assigned a value of 1. (F) The number of α SMA⁺ myofibroblasts in the dermis was determined by counting cells. Representative images of α SMA staining in the skin. Values were determined in six random microscopic fields in $n = 4-6$ mice per groups. * $P < 0.05$, ** $P < 0.01$. Scale bar = 50 μ m.

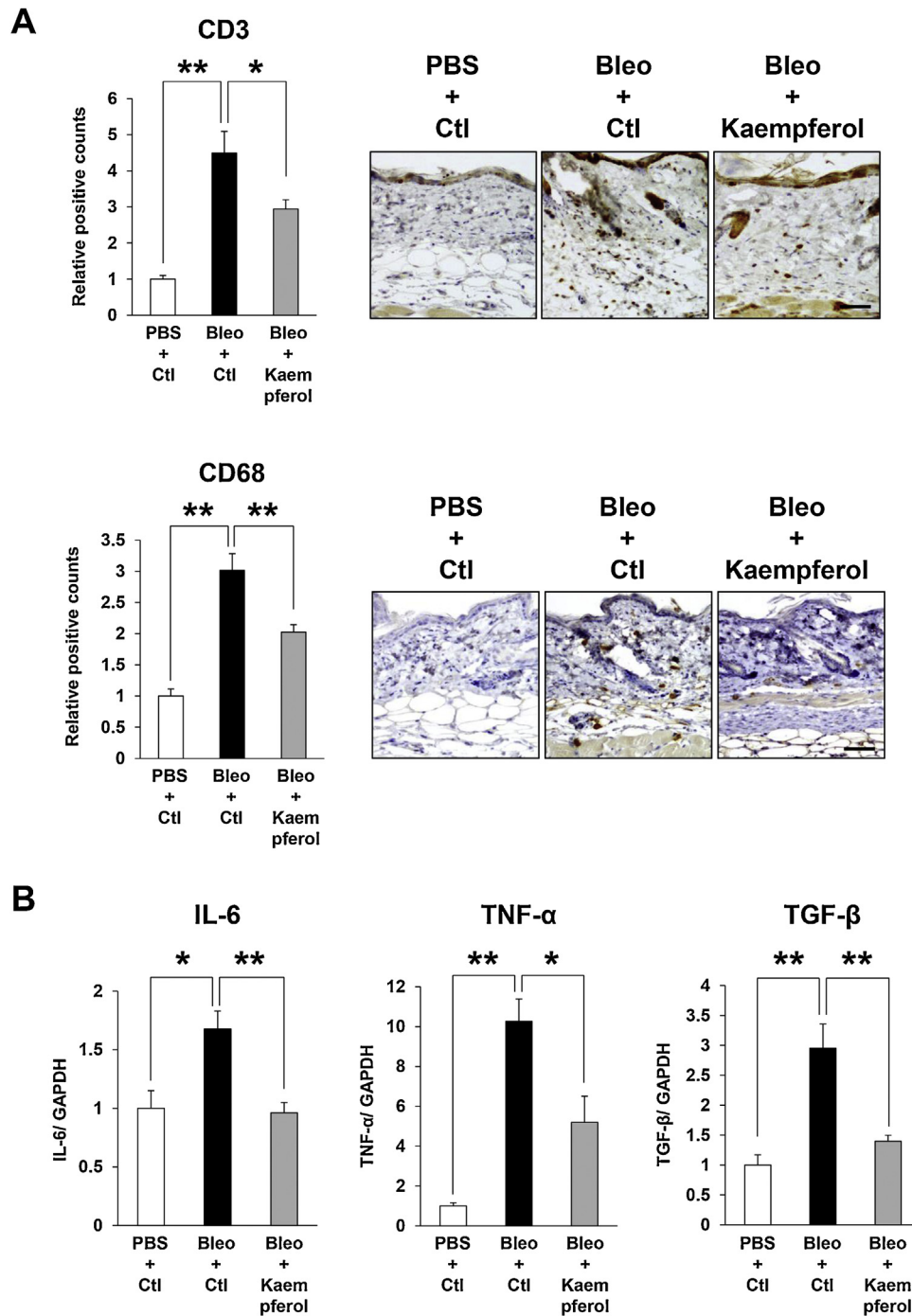


Fig. 3. Kaempferol suppressed the amount of inflammatory cells and the inflammatory and pro-fibrotic cytokines in the skin of bleomycin-treated mice *in vivo*. (A) The number of infiltrating CD3⁺ T cells and CD68⁺ macrophages in the dermis was determined by counting cells. Representative images of CD3 and CD68 staining in the skin. Values were determined in six random microscopic fields in $n = 4-6$ mice per groups. Scale bar = 50 μ m. (B) mRNA levels of inflammatory and pro-fibrotic cytokines, IL-6, TNF- α and TGF- β in the skin treated with subcutaneous injections of bleomycin of control PBS, and treated with intraperitoneal injection of kaempferol or control DMSO for 5 days. $n = 4-8$ mice in each group. The number of cells or mRNA levels in control mice were assigned values of 1. Values represent mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

inhibited by kaempferol treatment (Fig. 4E). These results suggested that kaempferol might reduce apoptotic cells *in vivo* as well as ROS production and oxidative stress-induced cell apoptosis *in vitro*.

4. Discussion

As oxidative stress impacts all aspects of the pathophysiology of SSc, it constitutes an interesting therapeutic target, and there is increasing evidence that the efficacy of anti-oxidative therapy is

very encouraging [7,8,35,36]. Yoshizaki et al. reported that edaravone, a free radical scavenger, has a significant inhibitory effect on fibrosis in SSc mouse model [7]. Piera-Velazquez et al. reported that selective NOX4 inhibitor exerted a highly effective anti-fibrotic effect in various animal models of tissue fibrosis [8]. Toyama et al. recently reported that dimethyl fumarate (DMF), an FDA-approved anti-oxidative and anti-inflammatory agent, was shown to have a beneficial effect for skin fibrosis in bleomycin-induced fibrosis mice model [35]. Polyphenols are natural antioxidants present in many plant foods, and among them,

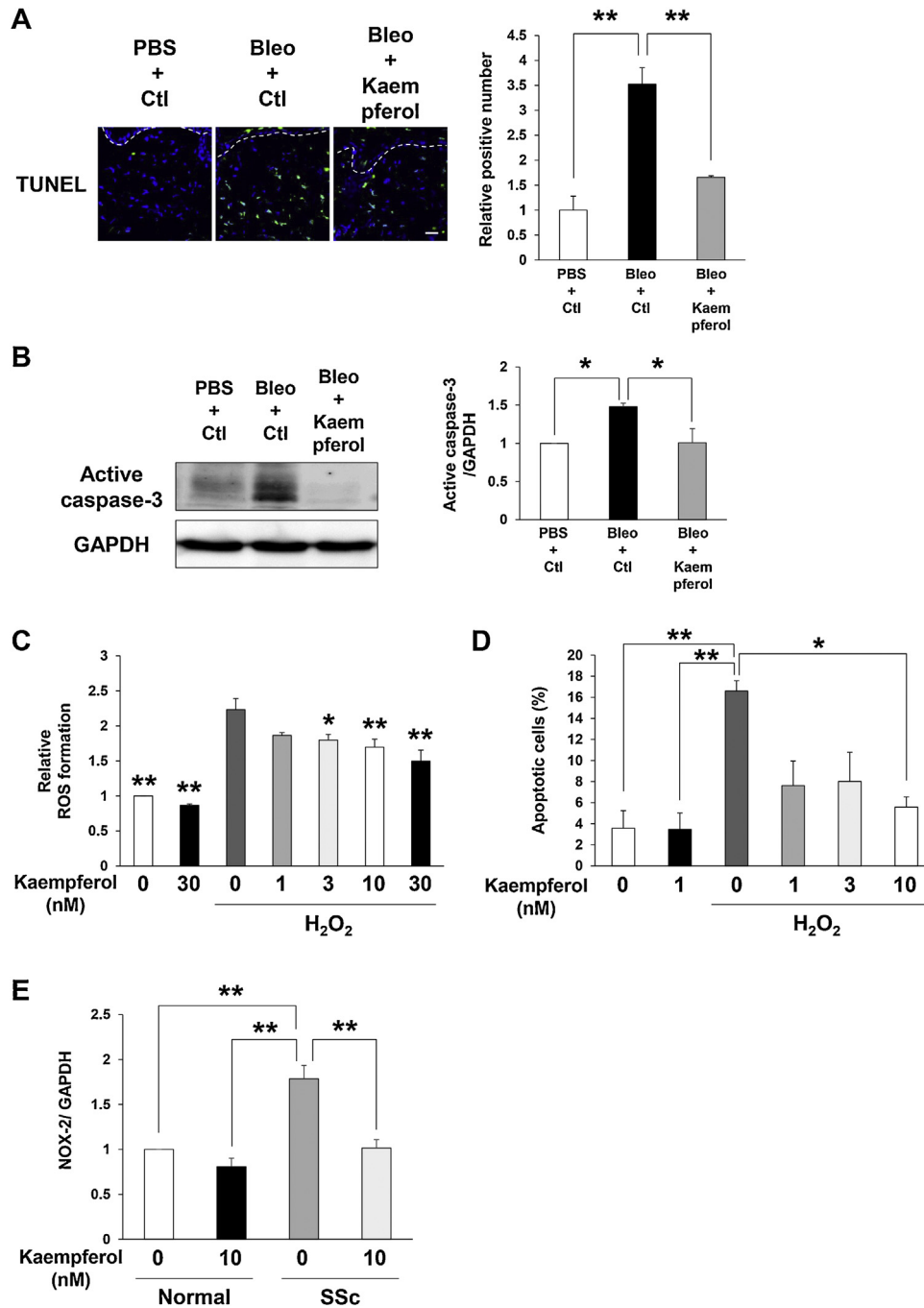


Fig. 4. Kaempferol reduced bleomycin-induced apoptosis *in vivo* and inhibited the oxidant-induced intracellular accumulation of ROS and apoptosis and necrosis in SSc fibroblasts *in vitro*. (A) After 5 days injection of bleomycin, the number of apoptotic cells in bleomycin-injected skin was determined by counting both TUNEL and DAPI positive cells. Values were determined in 8–9 random microscopic fields in $n = 3$ mice per groups. The number of apoptotic cells in control mice was assigned a value of 1. Scale bar = 20 μm . (B) After 5 days injection of bleomycin, the amount of active caspase-3 in the control PBS or bleomycin-injected skin treated with or without kaempferol was examined by immunoblotting. $n = 3$ mice per groups. Quantification of relative levels of active caspase-3 were accomplished via densitometry using ImageJ. (C) Quantification of H₂O₂-induced intracellular ROS production in SSc fibroblasts treated with or without kaempferol. ROS formation in cells without treatment was assigned a value of 1. $n = 6$ in each group. (** $P < 0.01$, * $P < 0.05$ relative to cells treated with H₂O₂ and without kaempferol) (D) Quantitation of the total amount of apoptotic cells (Annexin V⁺ and 7-AAD⁺) in SSc fibroblasts with or without H₂O₂ and/or kaempferol. (E) mRNA level of NOX2 expression in normal and SSc fibroblasts treated with or without kaempferol. $n = 3$. Values represent mean $\pm$ SEM. ** $P < 0.01$, * $P < 0.05$.

epigallocatechin-3-gallate (EGCG) present in green tea extracts is a scavenger of free radicals and is effective in decreasing oxidative stress in SSc [36].

Kaempferol is a natural flavonoid, and the anti-inflammatory effect of kaempferol has been demonstrated in several inflammatory diseases in animal models. For example, kaempferol markedly reduced the symptoms of lipopolysaccharide (LPS)-induced mastitis in mice and inhibited the expressions of

inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β [37]. Regarding the anti-oxidative effect of kaempferol, kaempferol can act as a potent scavenger of free radicals and superoxide radicals, resulting in the prevention and treatment of oxidative stress [10–13]. It has been reported that kaempferol inhibited cytokine- or glutamate-induced ROS production in human umbilical vein endothelial cells or neuronal cells, respectively [11,12]. Additionally, the diethylnitrosamine-induced reduction

of mRNA expression of antioxidant enzymes, such as catalase, glutathione peroxidase, and glutathione-S-transferase, can be restored by kaempferol [13]. Based on these findings, we hypothesized that kaempferol might suppress the fibrosis of skin in bleomycin-induced scleroderma mice model. In the present study, for the first time, we have shown that the intraperitoneal injection of kaempferol significantly suppressed bleomycin-induced skin fibrosis, as well as bleomycin-induced oxidative stress, and inflammation *in vivo*.

In SSc, the fibrotic process is thought to be initiated by vascular injury with ischemia-reperfusion phenomena which is accompanied by overactivation of NOX in ECs and a subsequent release of ROS [38]. Fibroblasts from patients with SSc constitutively produce a greater amount of ROS, such as H_2O_2 , through NOX activation, and ROS acts as signaling molecules and increases gene expressions of collagen type I and α SMA [39,40]. Consistent with these findings, we identified that the amount of collagen and the number of α SMA⁺ myofibroblasts in the lesional skin in bleomycin-treated mice were significantly reduced by kaempferol injection.

We have shown for the first time that oxidative stress was increased at skin fibrotic lesion induced by bleomycin using a visualization model. Furthermore, we confirmed that oxidative stress was reduced by kaempferol injection. NOX2 appears to be the most widely distributed among NOX isoforms, such as neutrophils, macrophages, ECs, fibroblasts, and hematopoietic cells. NOX2 is crucial for the differentiation of monocytes to macrophages and for the polarization of M2 profibrotic type macrophages [41]. In addition, it has been reported that NOX2 contributes to ROS generation in SSc fibroblasts, playing a critical role in cell activation, including expressions of collagen type I and α SMA [34]. In our results, mRNA expressions of NOX2 were significantly enhanced by bleomycin treatment, and bleomycin-induced mRNA expressions of NOX2 were significantly inhibited by the administration of kaempferol, suggesting that the regulation of NOX2 by kaempferol might be associated with the regulation of the fibrotic responses. However, Trx2 mRNA levels in the skin treated with bleomycin were not changed by kaempferol treatment. We

also found that bleomycin and kaempferol treatment significantly increased the expression of Trx2 in the skin compared to control. However, it is difficult to interpret this phenomenon, and further investigation is warranted.

Increased production of ROS is responsible for the activation of lymphocytes, macrophages, and fibroblasts, and triggers the production of pro-inflammatory cytokines from these cells, such as IL-1 β , TNF- α , and IL-6 [42]. Additionally, it has been reported that TGF- β increased oxidative stress by reducing the antioxidant synthesis and promoting ROS production [43]. We identified that kaempferol treatment significantly reduced the number of CD3⁺ T cells and CD68⁺ macrophages, as well as mRNA expression levels of inflammatory and pro-fibrotic cytokines, including IL-6, TNF- α , and TGF- β in the lesional skin, suggesting that kaempferol might suppress the production of ROS, resulting in the suppression of the infiltration of inflammatory cells and the inflammatory and pro-fibrotic cytokine production in the lesional skin of bleomycin-treated mice *in vivo*.

Increased ROS production in the skin causes oxidative stress, which can lead to apoptosis. It has been reported that oxidative stress-induced apoptotic cells were increased in the bleomycin-induced fibrotic skin, and transplantation of mesenchymal stem cells overexpressing Trx-1 significantly inhibited oxidative stress and dermal fibrosis induced by bleomycin [44]. In addition, SSc fibroblasts were extremely sensitive to oxidative stress-induced apoptosis [39]. Similar to these previous results, we demonstrated that kaempferol injection reduced apoptotic cells in bleomycin-treated skin in mice *in vivo* as well as ROS production and oxidative stress-induced cell apoptosis in SSc fibroblasts *in vitro*.

On the basis of our results, we propose a model of the mechanism by which kaempferol suppresses skin fibrosis in bleomycin-induced SSc mouse model (Fig. 5). In the lesional skin of bleomycin-treated mice, vascular injury-induced hypoxia might increase ROS production, leading to the activation of fibroblasts, T cells, and macrophages, and subsequently increase collagen production from fibroblasts. Kaempferol injection might significantly reduce the amount of ROS, the number of myofibroblasts, T-cells, macrophages, and inflammatory and pro-fibrotic

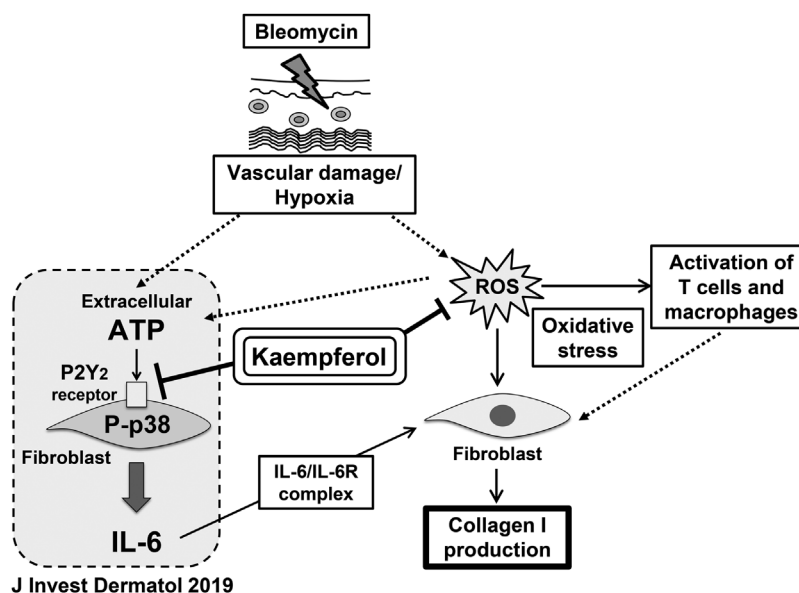


Fig. 5. Model of the mechanism by which kaempferol suppresses skin fibrosis in the bleomycin-induced SSc mouse model. In the lesional skin of bleomycin-treated mice, vascular injury-induced hypoxia might increase ROS production, leading to the activation of fibroblasts, T cells and macrophages, and subsequently increase collagen production from fibroblasts. Kaempferol injection might significantly reduce the amount of ROS, the number of myofibroblasts, T-cells and macrophages, and inflammatory and pro-fibrotic cytokines, including IL-6, TGF- β and TNF α in lesional skin. These beneficial effect of kaempferol might improve bleomycin-induced skin fibrosis. Additionally, kaempferol also inhibits extracellular ATP-induced IL-6/collagen type I production *in vivo* by the inhibition of ATP:P2Y₂ receptor signaling.

cytokines, including IL-6, TGF- β , and TNF α in the lesional skin. These beneficial effects of kaempferol might improve bleomycin-induced skin fibrosis.

In addition, kaempferol has a function of purinergic P2Y₂ receptor antagonist [45,46]. P2Y₂ receptor is one of the receptors of extracellular adenosine triphosphate (ATP) [47]. ATP, the ubiquitous source of energy in cells, can be released from cells by various stimulations, such as mechanical stress, tissue injury, inflammation, and hypoxia [47–49]. Recent evidence suggests that extracellular ATP can serve as damage-associated molecular patterns (DAMPs), which initiates an inflammatory response by autocrine/paracrine signaling [49]. The released ATP is recognized by plasma membrane-localized purinergic receptors, such as P2X and P2Y [47]. P2X receptors are ATP-gated ion channels, and P2Y receptor is a G protein-coupled receptor [47]. We recently identified that hypoxia enhanced ATP release, and extracellular ATP-induced phosphorylation of p38 via P2Y₂ receptor enhanced IL-6 and collagen type I production in SSc fibroblasts [50]. Our previous findings suggested that kaempferol also might inhibit extracellular ATP-induced IL-6/collagen type I production *in vivo* by the inhibition of ATP:P2Y₂ receptor signaling.

In conclusion, kaempferol inhibited collagen production via through reducing oxidative stress, inflammation, and oxidative cellular damage in bleomycin-induced skin fibrosis model. In addition, kaempferol might inhibit ATP-induced IL-6 production, leading to a decrease in collagen production in this model. This study suggests that kaempferol could be an alternative treatment for skin sclerosis in patients with SSc. Because it has been considered that oxidative stress injury might be associated with the vasculopathy [3,4,20,21], our results suggest that kaempferol also may be effective for vasculopathy in SSc patients. However, further examination is required.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jdermsci.2019.08.004>.

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