The Potential of Twendee X® as a Safe Antioxidant Treatment for Systemic Sclerosis

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Article

The Potential of Twendee X® as a Safe Antioxidant Treatment for Systemic Sclerosis

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Abstracts: Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by debilitating symptoms including skin and visceral fibrosis, systemic skin hardening, Raynaud's phenomenon and other vascular dysfunctions, and immune abnormalities. The disease poses a significant medical challenge with no cure currently available. As such, SSc is designated as an intractable disease in Japan, with patients receiving government subsidies for medical expenses. Oxidative stress (OS) is implicated in the pathogenesis of SSc and the antioxidant combination Tweendee X® (TwX) has shown promising results in mitigating OS-related conditions. HOCl-induced SSc-mice are known to exhibit similar skin and visceral fibrosis, vascular disorders, and autoimmunity observed in human. In a study done using these mice, Twx demonstrated a significant reduction in lung and skin fibrosis compared to untreated HOCl-induced SSc mice. TwX also significantly reduced advanced oxidized protein products (AOPP) in serum, Col-1 gene expression and the activation of B cells involved in autoimmunity. SSc is a disease state in which autoimmune abnormalities, fibrosis, and vascular disorders are intertwined, and therefore for a treatment to be effective it needs to address multiple issues. Thus the efficacy of TwX may stem from its strong antioxidant capacity to regulate OS, which cannot be achieved by a single antioxidant on its own. In other studies, targeting other OS-related conditions, TwX was safe and proved to be effective at prevent dementia in humans with mild cognitive impairment and to significantly improve cognitive impairment in Alzheimer's (AD) mouse models. TwX may offer a new approach to antioxidant treatment for SSc, to address multiple abnormalities that are characteristic of the disease.

Keywords: Oxidative stress; Twendee X®; Antioxidant; Systemic sclerosis; autoimmunity

1. Introduction

Systemic sclerosis (SSc) is an autoimmune disease of unknown cause, characterized by skin and visceral fibrosis, vascular dysfunction, and immune dysfunction [1]. Although the mechanisms responsible for the clinical manifestations of the disease are unknown [2], the involvement of reactive oxygen species (ROS) in the pathogenesis of SSc is well documented [3–6]. Raynaud's phenomenon (RP) occurs in 90% of SSc patients, causing frequent episodes of hypoxia-reperfusion. This results in a positive feedback effect of luminal narrowing and ischemia that contributes to the generation of ROS and free radicals. This oxidative environment further exacerbates the disease by triggering endothelial damage, intimal thickening, and fibrosis. Such ischemia and reperfusion induce oxidative

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stress (OS) and activae antioxidant enzymes [7]. Furthermore, skin fibroblasts in SSc patients have been shown to elevate ROS, which triggers collagen synthesis [5], emphasizing the significant role of OS in SSc.

Twendee X® (TwX) is an antioxidant combination comprising eight active ingredients: vitamin C, L-glutamine, niacin, L-cystine, coenzyme Q10, vitamin B2, succinic acid and fumaric acid [8,9]. Despite being a dietary supplement, it has undergone and passed stringent safety tests required for pharmaceuticals including chromosomal aberration test, toxicity test, and mutation test..

TwX has demonstrated various beneficial effects including protecting mitochondria, increasing ATP production, reducing blood OS, maintaining autophagy and neurogenesis, and elongating telomeres [10]. The effect of TwX on cognitive function has also been studied in varying disease models. A multicenter randomized, double-blind, placebo-controlled intervention clinical trial has demonstrated its potential to prevent dementia in Japanese patients with mild cognitive impairment (MCI) [11]. Moreover, TwX has shown positive outcomes in mouse models of Alzheimer's (AD) with chronic cerebral hypo-perfusion (CCH+APP23 mice); motor coordination and working memory were improved and hippocampal neurons were restored. Other outcomes derived from TwX include significant improvement of cognitive impairment, reduction of Aβ pathology and neuronal loss, and alleviation of neural inflammation and OS [12,13].

In a mouse model of ischemic stroke, pretreatment with TwX (20 mg/kg/d) for 14 days not only reduced infarct size, but also decreased the expression of OS markers and tumor necrosis factor-α (TNF-α) and inflammation markers[14]. Furthermore, in mice with impaired cognitive function and coordination due to increased OS from vitamin E deficiency, TwX significantly improved cognition and coordination, while also resulting in significant increases in brain-derived neurotrophic factor and nerve growth factor levels [15]. Recent reports have also suggested the potential of TwX to enhance the quality of life in dementia patients by influencing gut microbiota [16]. As well, the findings from other studies on TwX support its potential to effectively regulating OS in SSc. The aim in this study is to investigate the effects of TwX on various human-like SSc symptoms in mice that were injected with HOCl, an oxidation accelerator, into their dermis [17,18].

2. Results and Discussion

2.1. Reduction of OS in HOCl-Induced SSc by TwX

Advanced oxidation protein products (AOPP) in the sera of HOCl-induced SSc mice were significantly elevated compared to the control group treated with PBS (PBS control) (p=0.02, Figure 1). In contrast, HOCl-induced SSc mice treated with TwX (20 mg/kg/d) (HOCl+TwX mice) had significantly decreased AOPP levels in the sera (*p<0.05, Figure 1).

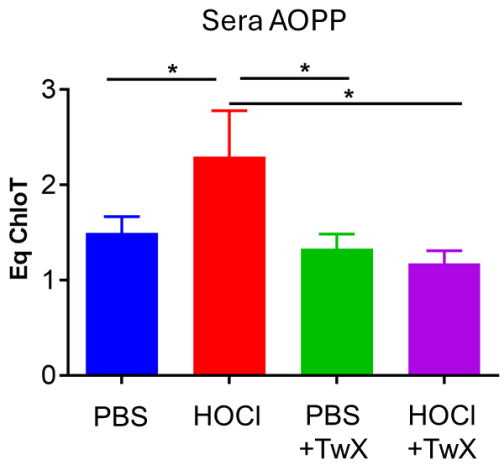

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Figure 1. Effect of Twendee X® on sera redox status. Concentrations of advanced oxidation protein products (AOPP) in the sera from mice (mM of chloramine T equivalent). Each box represents mean ± SEM from n = 10 biologically independent mice. The comparison by one-way ANOVA is followed by a Bonferroni test. ∗P ≤ 0.05.

Serum AOPP concentrations in SSc patients are usually higher than in healthy subjects [3]. This was also observed in the present study; HOCl-induced SSc mice had significantly higher AOPP than PBS-treated mice ($p < 0.05$). AOPP is a marker of OS. Among ROS, H2O2 can activate fibroblast proliferation [3]. HOCl-induced ROS and AOPP are said to activate both endothelial cells and fibroblasts, and a dose-dependent proliferative response can be observed. In fact, serum from SSc patients with RP shows significantly higher levels of H_2O_2 and proliferation of NIH3T3 fibroblasts compared to serum from SSc patients without RP and healthy subjects [3]. Additionally, decreased concentrations of antioxidants such as ascorbic acid, α-tocopherol, and β-carotene, as well as lower selenium levels have been reported in SSc patients [7]. This increase in ROS and deficiency in antioxidant capacity increases OS and contributes to the development of free radical-mediated damage. This elevated OS is further exacerbated not only by inflammatory processes but also by frequent reperfusion injury such as that seen in RP. Vascular changes including microvascular endothelial injury, vasospasm tendency with inadequate vasodilatory response, changes in the coagulation/fibrinolytic system, and proliferation of intimal cells leading to microvascular system occlusion are significant events in the early stages of SSc, and it highlight the crucial role of OS in the early stages of SSc [19] and in the progression and worsening of the disease.

Based on the above, at a certain point reducing OS is on of the pathway able to alleviate various symptoms of SSc. TwX has been shown to protect cells and mitochondria by lowering cellular and mitochondrial ROS levels and increasing Mn-SOD and Cu/Zn-SOD activity[10]. These effects significantly suppress OS caused by blood hydro-peroxides and other substances in CCH+APP23 mice and in OPP rats treated with orthophenyl phenol (OPP), a fruit and other preservative that induces ROS in the body [12,13,16]. In the present study, TwX affected OS to significantly reduced HOCl-derived oxidative damage.

2.2. Inhibition of HOCl-Induced Skin and Lung Fibrosis by TwX

Continuous intradermal injection of HOCl solution into mice replicates human SSc, inducing local and systemic fibrosis, inflammation, autoimmunity, and vascular injury [18]. The skin thickness (Figure 2A) and hydroxyproline (OH proline) levels in the skin (Figure 2B) of HOCl-induced SSc mice was significantly higher than those in the control group treated with PBS ($p = 0.002$ for dermal thickness and $p = 0.0037$ for OH proline concentration). This indicated elevated collagen levels and increased Col1 gene expression ($p<0.0001$, Figure 2D). However, treating these mice with TwX (20 mg/kg/d) significantly suppressed OH proline levels ($p <0.05$ for the skin, $p<0.001$ for the lung) and Col1 mRNA expression ($p<0.001$), thereby suppressing collagen accumulation (Figure 2B–D).

Further observations, confirmed by histopathological images, showed that skin thickness induced by HOCl was gradually suppressed from day 14 onward, and significantly reduced to a thickness similar to that of PBS+TwX mice from day 20 onward ($p < 0.05$, Figure 2A,E). Similarly, the lungs of HOCl-induced SSc mice showed high concentrations of OH proline, ($p = 0.017$, Figure 2C), and TwX significantly reduced this collagen accumulation to lower levels than in PBS control mice.

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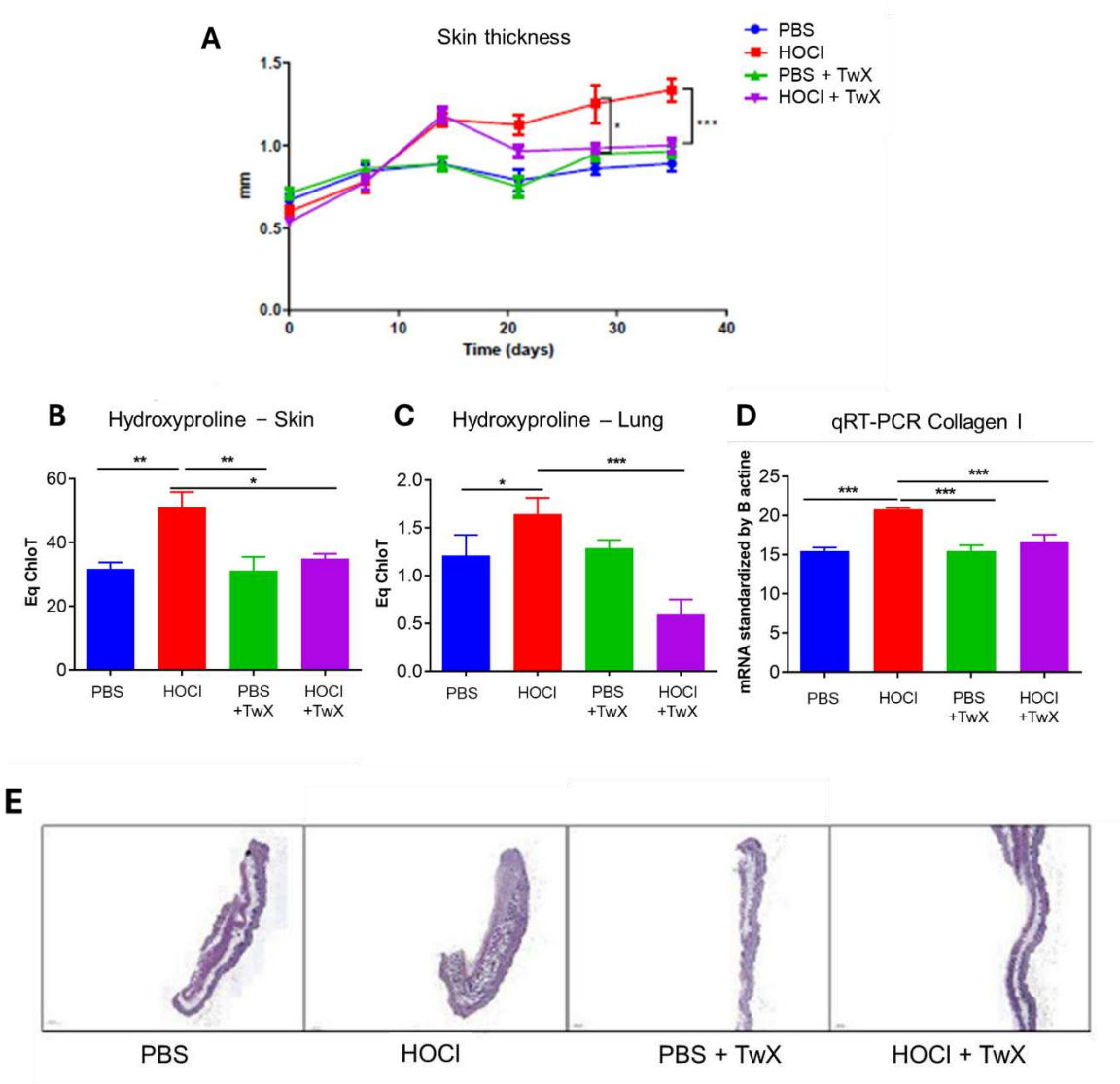

Figure 2. Effect of Twendee X® on fibrosis parameters. (A) Change of skin fold thickness in millimeters from day 1 to day 36, measured weekly. (B) Collagen type I levels in skin (mg/punch biopsy) and (C) in lung (mg/lobe biopsy) were evaluated by Hydroxyproline dosage. Each box represents mean ± SEM from n = 10 individual mice. (D) Relative mRNA level of Collagen-1. Results were standardized by GAPDH, derived from n = 10 biologically independent mice. (E) Representative H&E dyed skin sections of 6µm, showing enhanced fibrosis in mice. Photographs were taken with a Nikon Eclipse 80i microscope. Original magnification ×20. The scale represents 200µm. Data are expressed as the mean ± SEM. Statistical analysis of the different sample groups were carried out by one-way ANOVA followed by a Turkey multi comparison test at each time point (A), or by a Bonferroni test (B-D). ∗P ≤ 0.05; ∗∗P ≤ 0.01; ∗∗∗P ≤ 0.001.

Several human skin pathologies, including SSc, are associated with significant redox imbalance at the cellular level [3,20,21]. Antioxidant administration has also been reported to suppress skin and pulmonary fibrosis in SSc [22,23]. One example is Edaravone, a novel, free radical scavenger, and neuroprotective agent used in the treatment of acute embolic stroke in humans [24]. The skin fibrosis score in bleomycin-induced mouse models (BLM mice) increases over time, and while Edaravone significantly reduced dermal skin thickness it did not lower to the level of the control mice [22]. TwX also had an effect on skin and lung fibrosis in HOCl-induced SSc mice. Although, the effect of TwX on dermal thickening was not observed in the early stages of HOCl treatment, the effect emerged gradually and eventually decreased dermal thickness to a state close to that of the PBS control mice.

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Although TwX also has free radical scavenging [25] and neuroprotective effects [10], it may be necessary to use more immediate agents to improve skin thickening from the initial stage. OS scavenging could lead to a rational targeted therapeutic approach for SSc, however, the complexity and repetitive nature of the cascading system in SSc makes it impossible to achieve efficacy with a treatment derived from a single antioxidant [7]. The composition of TwX, which includes eight high efficacy antioxidants, may underlie its effectiveness in alleviating SSc symptoms to a state similar to the PBS control mice. The same could be true for collagen content in skin and lungs.

The expression of α-SMA protein was significantly enhanced in HOCl-induced SSc mice ($p<0.05$ Figure 3A). However, TwX significantly reduced it to a level similar to that of PBS control mice ($p=0.021$, Figure 3A).

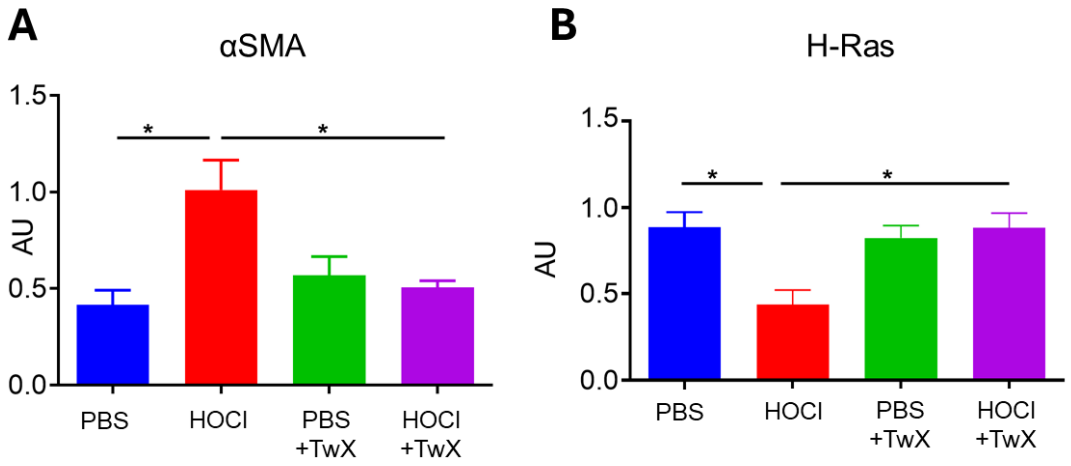

Figure 3. Effect of Twendee X® on fibroblast differentiation. (A) α-SMA and (B) H-Ras, in skin were evaluated by Western Blot. Results were standardized by tubulin, derived from n = 10 biologically independent mice. The comparison by one-way ANOVA is followed by a Bonferroni test. $*P \leq 0.05$.

α-SMA is expressed primarily in vascular smooth muscle and is involved in the differentiation of fibroblasts into myofibroblasts, which are responsible for the production of extracellular matrix in fibrotic diseases such as SSc. It has been reported that the development of ROS in SSc fibroblasts increases the expression of type 1 collagen and α-SMA genes [26], while the expression of type I collagen and α-SMA genes also activate ROS [5,27]. TwX was shown to inhibit SSc fibrosis by reducing α-SMA as well as ROS.

In contrast, the expression of H-Ras protein was significantly reduced in HOCl-induced SSc mice ($p<0.05$, Figure3B). H-Ras proteins are primarily involved in the regulation of cell division. When the protein binds to GDP, it does not relay signals to the cell's nucleus to divide. H-Ras-GTPase are activated for example during renal fibrosis and play crucial roles in regulating both cell proliferation and TGF-β-induced epithelial-mesenchymal transition [28]. The results suggest that normal homeostatic functions may act to protect cells from HOCl-induced excessive division, resulting in a decrease in H-Ras. On the other hand, this decrease was not observed in PBS control or HOCl+TwX mice, likely due to the absence of induced fibrosis. We speculate that TwX prevented fibrosis before normal homeostatic function was activated in H-Ras.

2.3. Effects on Inflammation and Immunity in SSc

HOCl-induced SSc mice have been reported to exhibit systemic immune cell infiltration and release of inflammatory mediators [29]. Although the expression of Il-6, Il-33, and Il-17 mRNA tend to be higher in HOCl-induced SSc mice in this model, they did not show significant changes from PBS control mice in this study. A similar trend was observed after TwX treatment, however a decreasing trend mRNA expression was observed (Figure 4A–C).

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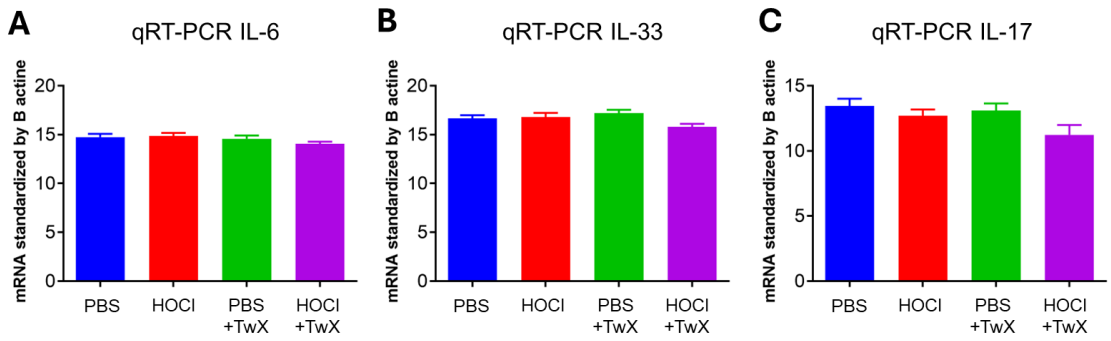

Figure 4. Effect of Twendee X® on Collagen and cytokines expression. Relative mRNA level of (A) Il-6, (B) Il-33, and (C) Il-17 mRNA levels in skin evaluated by qRT-PCR. Results were standardized by GAPDH, derived from n = 10 biologically independent mice per. The comparison by ANOVA is followed by a Bonferroni test. No significant difference was observed.

In the present study, there was no significant increase in the expression of inflammatory cytokines in HOCl-induced SSc mice. However, since TwX has been shown to suppress inflammation in CCH+APP23 mice [12,13], it may have done the same in SSc.

SSc is a systemic autoimmune disease with chronic activation of adaptive immunity by nuclear autoantigens. In HOCl-induced SSc mice, activation of splenic B cells and CD4+ T cells was increased compared to PBS control mice. TwX significantly reduced these activations ($p<0.001$ (CD40, MHCII and CD69), $p<0.01$(CD44), Figure 5A–D).

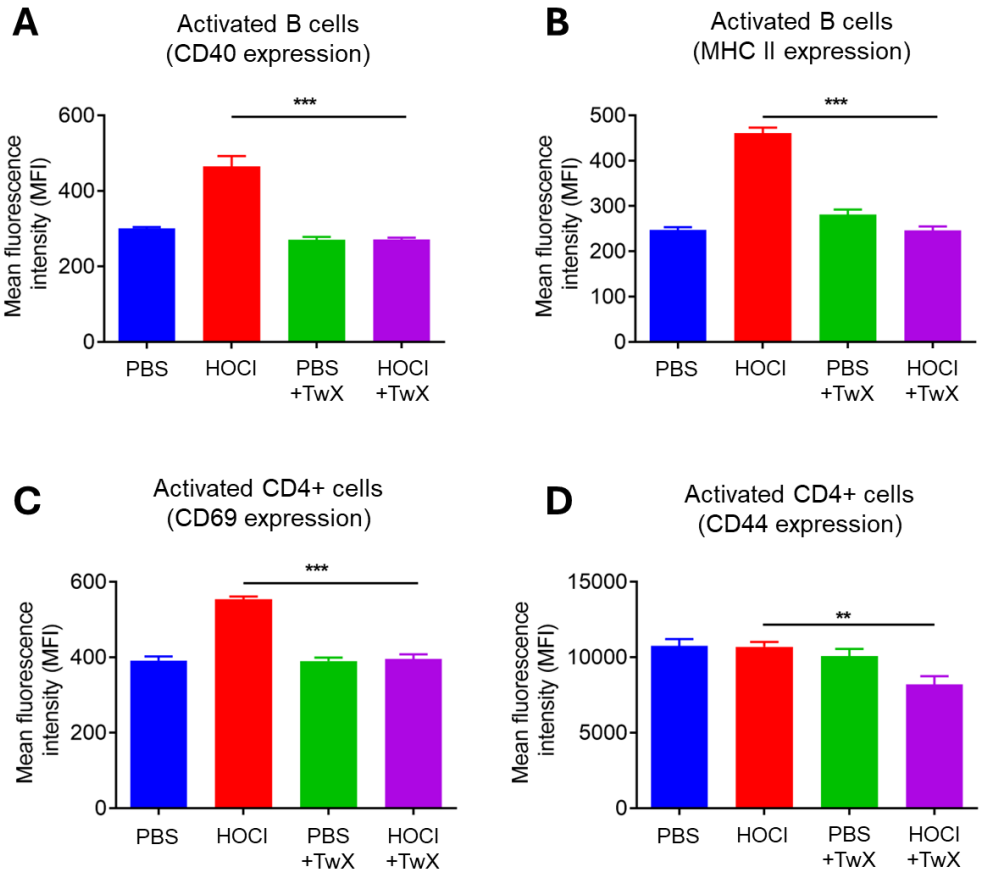

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Figure 5. Effects of Twendee X® on B and T CD4+ cells activation assessed by flow cytometry in SSc mice. The side scatter (SSC) and the forward scatter channels (FSC) were used to gate the leukocytes. A total of 100,000 events were accumulated for each sample. Doublets were excluded with FSC-A and FSC-H channels. (A and B) Activation of B220+ cells assessed by CD40 expression (A) and MHC II expression (B). (C and D) Activation of CD4+ T cells assessed by CD69 (C) and CD44 (D) expression. Data represent the mean fluorescence index (MFI) and SEM from 10 mice per group. Statistics are realized after a significant one-way ANOVA. A Kruskal-Wallis test was used to detect significant differences between PBS and HOCl groups first and then HOCl and HOCl+TwX groups. **p≤ 0.01; ***p≤0.001.

Activated macrophages play a key role in triggering and perpetuating the inflammatory process and in developing fibrosis during SSc. No significant difference was observed in the frequency of splenic macrophages between the sample groups (Figure 6A). The expression of CD86 and Ly6C showed an increasing trend on macrophages of HOCl-induced SSc mice compared to controls (Not significant, Figure 6B,D); in the expression of CD206, there was a significant increase compared to controls (p<0.001, Figure 6C). Interestingly, HOCl+TwX mice showed a significant decrease in CD86 and CD206 expression in their splenic macrophages (p=0.009 and p<0.001, respectively, Figure 6B,C), while Ly6C expression was notably increased (p<0.01, Figure 6D). In addition, we observed a polarization toward the M2 profibrotic macrophage phenotype (p<0.001, Figure 6E) in HOCl-induced SSc mice that was reversed in the HOCl+TwX mice (p<0.001).

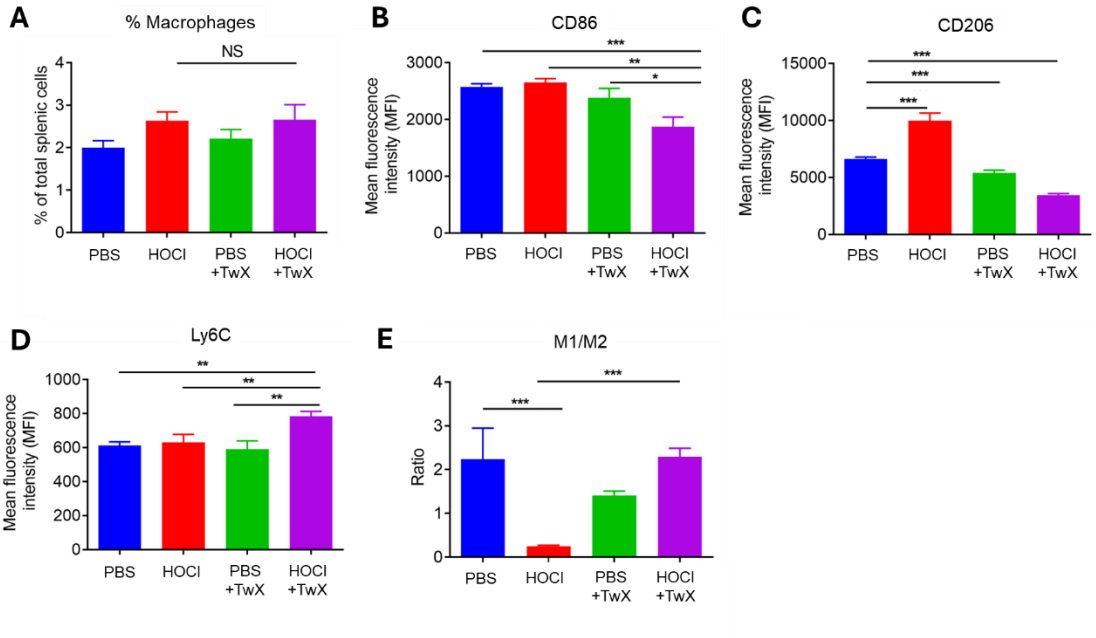

Figure 6. Flow cytometric analysis of the effects of Twendee X® on splenic macrophage phenotype in SSc mice. Splenic macrophages were gated on CD11b and F4/80 positive cells among CD45 positive. A total of 100,000 events were accumulated for each sample. Doublets were excluded with FSC-A and FSC-H channels. (A) Percentage of splenic macrophages among total splenic cells. Data represent the percentage and SEM. (B-D) Flow cytometric analysis of CD86, Ly6C and CD206 expression on splenic macrophages. Data represent the mean fluorescence index (MFI) and SEM from 10 mice per group. (E) Ratio of M1/M2 macrophages frequency. M1 macrophages were defined as B220−F4/80+CD11b+Ly6CHighCD206- and M2 macrophages as B220−F4/80+CD11b+Ly6cLowCD206+. Statistics are based on a significant one-way ANOVA. A Kruskal-Wallis test was used to detect significant differences between PBS and HOCl groups first and then HOCl and HOCl+TwX groups. NS: Not significant; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

In SSc, macrophages play a important role in the disease pathogenesis. When macrophages are activated, they release mediators and express surface markers, and normally the activation of both

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alternative macrophages (M2) and inflammatory macrophages (M1) is associated with SSc [30]. Macrophages are the predominant immune cell population in SSc pathology, and their dysfunction leads to abnormal repair and regeneration with runaway inflammatory mediators and growth factors [31]. Excessive accumulation of M2 macrophages is closely associated with fibrosis [32,33], which results from the abnormal accumulation of extracellular matrix (ECM) components such as collagen and fibronectin. ECM promotes wound healing and tissue repair in mild tissue injury, however, in severe injury, excessive accumulation of ECM can disrupt tissue structure and lead to organ dysfunction [34]. Thus, macrophages, play a crucial role in fibrosis pathogenesis [35,36], especially M2a macrophages, which significantly promote fibrosis progression [33,37]. The present study suggests that a shift in macrophage balance toward the M2 phenotype occurred during the chronic phase (after the 21st day of the experiment) in HOCl-induced SSc mice. However, the trend was reversed when TwX was administered, resulting in the accumulation of M1 macrophages over M2 macrophages, thereby slowing the fibrotic process.

3. Materials and Methods

3.1. Materials

TwX is a compound consisting of the following active ingredients: L-glutamine (34.6 wt%), ascorbic acid (34.2 wt%), L-cystine (18.2 wt%), coenzyme Q10 (3.6 wt%), succinic acid (3.6 wt%), fumaric acid (3.6 wt%), riboflavin (1.5 wt%), and niacin amid (0.7 wt%). TwX mixture was dissolved in sterile water and stored at 4 °C until use.

3.2. Chemically Inducing SSc in Mice In Vivo

The animal protocol for this study was reviewed and approved by the local Ethic committee (Comité d'Ethique en matière d'Expérimentation animale Paris Descartes CEEA34, Animal facilities C75-14-05, Agreement DAP 2019080816497350 #26065). Six week-old female BALB/c mice were purchased from Janvier Labs (Le Genest-Saoint-Isle, France). Mice were randomly distributed into experimental and PBS control groups (n = 10 per group). The experimental groups included the HOCl-induced SSc mice group (HOCl), the group given PBS and TwX (PBS+TwX), and the TwX-treated HOCl-induced SSc group (HOCl+TwX).

Subcutaneous injections of 200µl of HOCl into the back of the mice were administered once daily for 6 weeks, as previously described [17,18]. The PBS control group and PBS+TwX group received injections of 100 µl of sterilized PBS.

One month before and during the 6 weeks of HOCl injections, the mice consumed plain tap water or water with TwX ad libitum(20mg/kg/d). Two days after the final injection, the animals were euthanized by cervical dislocation, and samples of sera, spleen, lungs and skin biopsies were collected.

3.3. Evaluating Fibrosis

Fibrosis of the skin was assessed weekly in vivo, by measuring the dermal thickness of the shaved backs of the mice. This assessment was conducted under double-blinded conditions using a caliper and expressed in millimeters once a week until the end of the experiment.

3.4. Collagen Content Measurement

The collagen content was assessed in the skin and lungs using the OH-proline content evaluation as recommended by Woessner [38]. Briefly, after completing punch biopsies (5-mm diameter), the samples were incubated in HCl (6 M) for 3 h at 120℃. The pH of the samples was adjusted to 7, and then mixed with chloramine T (0.06 M) and incubated for 20 min at room temperature. Perchloric acid (3.15 M) and p-dimethylaminobenzaldehyde (20%) were then added and samples were incubated for an additional 20 min at 60℃. The absorbance was determined at 557 nm with a Fusion microplate spectrophotometer (PerkinElmer, Wellesley, MA, USA).

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3.5. Histopathologic Analysis

A 5-µm-thick tissue section was prepared from the mid-portion of paraffin-embedded lung and skin sample and stained with hematoxylin and eosin. Slides were examined by standard brightfield microscopy (Olympus BX60, Tokyo, Japan) by a pathologist who was blinded to the animal's group assignment. Assays of AOPP in sera were diluted (1:5) in PBS and distributed (200 µL) onto a 96-well plate with 10 µL of 1.16 M potassium iodide. Calibration used a two-fold dilution series of chloramine-T solution within a range of 0 to 100 mM. The absorbance was read at 340 nm on a microplate reader (Fusion; PerkinElmer, Wellesley, MA, USA) and AOPP concentration was expressed as mM of chloramine-T equivalents.

3.6. Evaluation of Fibroblasts

α-SMA and H-RAS proteins expressions in mouse skin were analyzed. Skin samples were mixed in RIPA lysis buffer. Proteins (30 µg per well) were subjected to 10% polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, blocked with 5% nonfat dry milk in TBS-T, then incubated overnight at 4°C with an anti-mouse α-SMA (Abcam, Cambridge, UK. ref ab5694) or an anti hRAS antibody (Santa Cruz Biotechnology, Dallas, TX, USA. ref sc-53959). The membranes were then washed, incubated with an HRP-conjugated antibody (Invitrogen, Carlsbad, CA, USA. goat anti rabbit ref A27036, goat anti mouse ref A28177). Tubuline and B actine were used to standardize the OD values obtained for the different samples.

3.7. Evaluation of mRNA Expressions

The qRT-PCR technique was conducted as follows: the skin samples taken from the four groups of mice were immediately frozen in liquid nitrogen. Total RNA was extracted from the mouse tissue using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

The amplified genes were col 1 (collagen 1) 1, Il-33, Il-6 and Il-17. The cDNA was synthesized using the Maxima H Minus cDNA Synthesis Master Mix kit (Thermo Fisher Scientific, Carlsbad, CA, USA). The qPCR was performed using the SensiFAST SYBR No-ROX kit (Bioline, Memphis, TN, USA). The transcription levels of the selected genes were quantified in a LightCycler480® (Roche, San Francisco, CA, USA) thanks to calibration samples (serial dilution) and normalized to two reference gene (GAPDH). The primer sequences used for qPCR are as follows: mm-Col1a1_F GCTCCTCTTAGGGGCCACT, mm-Col1a1_R CCACGTCTCACCATTGGGG, IL-17_F CTG CTG AGC CTG GCG GCT AC, IL-17_R CAT TGC GGT GGA GAG TCC AGG G, IL-33_F ACT ATG AGT CTC CCT GTC CTG, IL-33_R GCT TCA AAG GGG TGA CGT, IL-6_F GAG GAT ACC ACT CCC AAC AGA CC, IL-6_R AAG TGC ATC ATC GTT GTT CAT ACA.

3.8. Evaluating Immune Status

Spleen cell suspensions were prepared after hypotonic lysis of erythrocytes in potassium acetate solution and three washes in complete RPMI medium. For each mouse, splenocytes were quantified using a Malassez counting chamber. Cells were then incubated with an antibody at 4 °C for 30 min in the dark in PBS with 2% normal FBS. Flow cytometry was performed using a FACS Fortessa II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), according to standard techniques. To characterize splenic cells, the monoclonal antibodies used spleen cell suspensions prepared after hypotonic lysis of erythrocytes in potassium acetate solution and three washes in complete RPMI medium. For each mouse, splenocytes were quantified using a Malassez counting chamber. Cells suspensions were incubated for 20 min with the following antibodies: CD3 FITC, CD4 APC Fire 750, CD8 BV 605, CD69 PEDazzle594, CD40 PercPCy5.5, B220 APC, CD44 BV650, CMH II eFluor450 (DAPI) (MIX A) and F4/80 BV711, CD11b PercP Cy5.5, CMH II eFluor 450 (DAPI), CD80 BV421, CD86 FITC, CD206 Alexa Fluor 647, Ly6C PECy7 (MIX B).

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3.9. Statistical Analysis

The results were analyzed with the GraphPad Prism9 program (version 8.3.0). The one-way test ANOVA was used to verify that the groups were comparable, followed by a multi comparison test (Bonferroni or turkey). The Kruskal-Wallis test has been used as an alternative to ANOVA in the event that the assumption of normality was not acceptable. A difference < 0.05 was considered significant.

4. Conclusions

The findings from this study, in conjunction with previous research, support TwX as a potential therapy for regulating oxidative stress and its promising effects in various diseases. In the case of SSc, the present results demonstrated that TwX has a beneficial and systemic effect in the HOCl mouse model in terms of addressing the multi-faceted symptom including fibrosis and other abnormalities indicative of the disease. Therefore, it can be concluded that TwX holds promise as a safe and effective antioxidant therapeutic intervention for SSc.

Author Contributions: Conceptualization, H.I. and C.N.; data curation, F.Y., C.N. and Y.H.; formal analysis, C.N., F.Y. and Y.H.; funding acquisition, H.I.; investigation, H.I., C.N. and F.Y.; methodology, C.N.; project administration, H.I., C.N. and F.Y.; resources, C.N., H.I. and F.Y.; supervision, H.I. and T.Y.; validation, F.Y. and C.N.; visualization, F.Y. and Y.H.; writing—original draft, F.Y. and C.N.; writing—review & editing, H.I., F.Y., T.Y., and C.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki. The animal protocol used in this study was reviewed and approved by the local Ethic committee (Comité d'Ethique en matière d'Expérimentation animale Paris Descartes CEEA34, Animal facilities C75-14-05, Agreement DAP 2019080816497350 #26065).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are available upon proper request.

Conflicts of Interest: F.Y., Y.H. and H.I. are employees of Gifu University. The Division of Antioxidant Research is a laboratory that has been established at the Life Science Research Center at Gifu University. based on a research fund from the TIMA Establishment (Liechtenstein). T.Y. is an advisor to TIMA Establishment (Liechtenstein). The sponsor had no control over the interpretation, writing, or publication of this work.

References

1. LeRoy, E.C.; Medsger, T.A. Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol. 2001, 28, 1573-1576.
2. Gabrielli, A.; Avvedimento, E.V.; Krieg, T. Scleroderma. N Engl J Med. 2009, 360, 1989-2003.
3. Servettaz, A.; Guilpain, P.; Goulvestre, C.; Chéreau, C.; Hercend, C.; Nicco, C.; Guillevin, L.; Weill, B.; Mouthon, L.; Batteux, F. Radical oxygen species production induced by advanced oxidation protein products predicts clinical evolution and response to treatment in systemic sclerosis. Ann Rheum Dis. 2007, 66, 1202-1209.
4. Simonini, G.; Cerinic, M.M.; Generini, S.; Zoppi, M.; Anichini, M.; Cesaretti, C.; Pignone, A.; Falcini, F.; Lotti, T.; Cagnoni, M. Oxidative stress in Systemic Sclerosis. Mol Cell Biochem. 1999, 196, 85-91.
5. Sambo, P.; Baroni, S.S.; Luchetti, M.; Paroncini, P.; Dusi, S.; Orlandini, G.; Gabrielli, A. Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. Arthritis Rheum. 2001, 44, 2653-2664.
6. Ogawa, F.; Shimizu, K.; Muroi, E.; Hara, T.; Hasegawa, M.; Takehara, K.; Sato, S. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. Rheumatology (Oxford). 2006, 45, 815-818.
7. Simonini, G.; Pignone, A.; Generini, S.; Falcini, F.; Cerinic, M.M. Emerging potentials for an antioxidant therapy as a new approach to the treatment of systemic sclerosis. Toxicology. 2000, 155, 1-15. doi: 10.1016/s0300-483x(00)00272-9.

8. Inufusa, H. Characterization of cell protection effects of Twendee X by oxidative stress. J World Mitochondria Soc. 2016, 2, 42.
9. Inufusa, H. Composition for protection against cytotoxic effects. TIMA Foundation. Patent No. 5777821, 2015-9-9.
10. You, F.; Harakawa, Y.; Yoshikawa, T.; Inufusa, H. Why Does the Antioxidant Complex Twendee X® Prevent Dementia? Int. J. Mol. Sci. 2023, 24, 13018. doi: 10.3390/ijms241613018.
11. Tadokoro, K.; Morihara, R.; Ohta, Y.; Hishikawa, N.; Kawano, S.; Sasaki, R.; Matsumoto, N.; Nomura, E.; Nakano, Y.; Takahashi, Y.; et al. Clinical Benefits of Antioxidative Supplement Twendee X for Mild Cognitive Impairment: A Multicenter, Randomized, Double-Blind, and Placebo-Controlled Prospective Interventional Study. J. Alzheimers Dis. 2019, 71, 1063–1069.
12. Liu, X.; Yamashita, T.; Shang, J.; Shi, X.; Morihara, R.; Huang, Y.; Sato, K.; Takemoto, M.; Hishikawa, N.; Ohta, Y.; et al. Clinical and Pathological Benefit of Twendee X in Alzheimer's Disease Transgenic Mice with Chronic Cerebral Hypoperfusion. J. Stroke Cerebrovasc. Dis. 2019, 28, 1993–2002.
13. Liu, X.; Yamashita, T.; Shang, J.; Shi, X.; Morihara, R.; Huang, Y.; Sato, K.; Takemoto, M.; Hishikawa, N.; Ohta, Y.; et al. Twendee X Ameliorates Phosphorylated Tau, α-Synuclein and Neurovascular Dysfunction in Alzheimer's Disease Transgenic Mice with Chronic Cerebral Hypoperfusion. J. Stroke Cerebrovasc. Dis. 2019, 28, 104310.
14. Kusaki, M.; Ohta, Y.; Inufusa, H.; Yamashita, T.; Morihara, R.; Nakano, Y.; Liu, X.; Shang, J.; Tian, F.; Fukui, Y.; et al. Neuroprotective Effects of a Novel Antioxidant Mixture Twendee X in Mouse Stroke Model. J. Stroke Cerebrovasc. Dis. 2017, 26, 1191–1196.
15. Fukui, K.; You, F.; Kato, Y.; Kimura, M.; Harakawa, Y.; Yoshikawa, T.; Inufusa, H. Twendee X, a mixed antioxidant supplement, improves cognitive function, coordination, and neurotrophic factor expression in long-term vitamin E-deficient mice. J. Clin. Biochem. Nutr. 2023, 72, 93–100. doi: 10.3164/jcbn.22-55.
16. You, F.; Harakawa, Y.; Yoshikawa, T.; Inufusa, H. Controlling Gut Microbiota by Twendee X® May Contribute to Dementia Prevention. Int J Mol Sci. 2023, 24,16642. doi: 10.3390/ijms242316642.
17. Servettaz, A.; Goulvestre, C.; Kavian, N.; Nicco, C.; Guilpain, P.; Chéreau, C.; Vuiblet, V.; Guillevin, L.; Mouthon, L.; Weill, B.; et al. Selective oxidation of DNA topoisomerase 1 induces systemic sclerosis in the mouse. J Immunol. 2009, 182, 5855-5864. doi: 10.4049/jimmunol0803705..
18. Batteux, F.; Kavian, N.; Servettaz, A. New insights on chemically induced animal models of systemic sclerosis. Curr Opin Rheumatol. 2011, 23, 511-518. doi: 10.1097/BOR.0b013e32834b1606.
19. Murrell, D.F. A radical proposal for the pathogenesis of scleroderma. J Am Acad Dermatol. 1993, 28, 78-85. doi: 10.1016/0190-9622(93)70014-k.
20. Amstad, P.; Peskin, A.; Shah, G.; Mirault, M.E.; Moret, R.; Zbinden, I.; Cerutti, P. The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. Biochemistry. 1991, 30, 9305-9313.
21. Marut, W.K.; Kavian, N.; Servettaz, A.; Nicco, C.; Ba, L.A.; Doering, M.; Chéreau, C.; Jacob, C.; Weill, B.; Batteux, F. The organotelluride catalyst $(PHTE)_2NQ$ prevents HOCl-induced systemic sclerosis in mouse. J Invest Dermatol. 2012, 132, 1125-1132.
22. Yoshizaki, A.; Yanaba, K.; Ogawa, A.; Iwata, Y.; Ogawa, F.; Takenaka, M.; Shimizu, K.; Asano, Y.; Kadono, T.; Sato, S. The specific free radical scavenger edaravone suppresses fibrosis in the bleomycin- induced and tight skin mouse models of systemic sclerosis. Arthritis Rheum. 2011; 63: 3086–3097.
23. Baral, H.; Sekiguchi, A.; Uchiyama, A.; Nisaa, Amalia, S.; Yamazaki, S.; Inoue, Y.; Yokoyama, Y.; Ogino, S.; Torii, R.; Hosoi, M.; et al. Inhibition of skin fibrosis in systemic sclerosis by botulinum toxin B via the suppression of oxidative stress. J Dermatol. 2021, 48, 1052-1061. doi: 10.1111/1346-8138.15888.
24. Edaravone Acute Infarction Study Group. Effect of a novel free radical scavenger, edaravone (MCI-186), on acute brain infarction: randomized, placebo-controlled, double-blind study at multicenters. Cerebrovasc Dis. 2003, 15, 222–229.
25. Feng, T.; Yamashita, T.; Tsunoda, K.; Matsumoto, N.; Tadokoro, K.; Sasaki R.; Abe K. In Vitro Free Radical Scavenging Activities of Dietary Supplements by Electron Spin Resonance. Brain Supplements. 2020, 2, 1-12.
26. Spadoni, T.; Svegliati, Baroni, S.; Amico, D.; Albani, L.; Moroncini, G.; Avvedimento, E.V.; Gabrielli, A. A reactive oxygen species-mediated loop maintains increased expression of NADPH oxidases 2 and 4 in skin fibroblasts from patients with systemic sclerosis. Arthritis Rheumatol. 2015, 67, 1611–1622.
27. Svegliati, S.; Cancello, R.; Sambo, P.; Luchetti, M.; Paroncini, P.; Orlandini, G.; Discepoli, G.; Paterno, R.; Santillo, M.; Cuozzo, C.; et al. Platelet-derived growth factor and reactive oxygen species regulate Ras protein levels in primary human fibroblasts via ERK1/2: amplification of ROS and Ras in systemic sclerosis fibroblasts. J Biol Chem. 2005, 280, 36474– 36482.
28. Grande, M.T.; Fuentes-Calvo, I.; Arévalo, M.; Heredia, F.; Santos, E.; Martínez-Salgado, C.; Rodríguez-Puyol, D.; Nieto, M.A.; López-Novoa, J.M. Deletion of H-Ras decreases renal fibrosis and myofibroblast activation following ureteral obstruction in mice. Kidney Int. 2010, 77, 509-518. doi: 10.1038/ki.2009.498.

29. Meng, M.; Tan, J.; Chen, W.; Du, Q.; Xie, B.; Wang, N.; Zhu, H.; Wang, K. The Fibrosis and Immunological Features of Hypochlorous Acid Induced Mouse Model of Systemic Sclerosis. Front Immunol. 2019, 10, 1861. doi: 10.3389/fimmu.2019.01861.
30. Peng, Y.; Zhou, M.; Yang, H.; Qu, R.; Qiu, Y.; Hao, J.; Bi, H.; Guo, D. Regulatory Mechanism of M1/M2 Macrophage Polarization in the Development of Autoimmune Diseases. Mediators Inflamm. 2023, 2023, 8821610. doi: 10.1155/2023/8821610.
31. Wynn, T. A.; Vannella, K. M. Macrophages in tissue repair, regeneration, and fibrosis. Immunity. 2016, 44, 450–462. doi: 10.1016/j.immuni.2016.02.015.
32. Chung, E. J.; Kwon, S.; Shankavaram, U.; White, A. O.; Das, S.; Citrin, D. E. Natural variation in macrophage polarization and function impact pneumocyte senescence and susceptibility to fibrosis. Aging. 2022, 14, 7692–7717. doi: 10.18632/aging.204309.
33. Jeljeli, M.; Riccio, L.G.C.; Doridot, L.; Chêne, C.; Nicco, C.; Chouzenoux, S.; Deletang, Q.; Allanore, Y.; Kavian, N.; Batteux, F. Trained immunity modulates inflammation-induced fibrosis. Nat Commun 2019, 10, 5670. https://doi.org/10.1038/s41467-019-13636-x
34. Henderson, N.C.; Rieder, F.; Wynn, T.A. Fibrosis: from mechanisms to medicines. Nature 2020, 587, 555–566. doi: 10.1038/s41586-020-2938-9.
35. Nakagawa, M.; Karim, M.R.; Izawa, T.; Kuwamura, M.; Yamate, J. Immunophenotypical characterization of M1/M2 macrophages and lymphocytes in cisplatin-induced rat progressive renal fibrosis. Cells. 2021, 10, 257. doi: 10.3390/cells10020257.
36. Chêne, C.; Rongvaux-Gaïda, D.; Thomas, M.; Rieger, F.; Nicco, C.; Batteux, F. Optimal combination of arsenic trioxide and copper ions to prevent autoimmunity in a murine HOCl-induced model of systemic sclerosis. Front Immunol. 2023, 14, 1149869. doi: 10.3389/fimmu.2023.1149869.
37. Duan, J.; Liu, X.; Wang, H.; Guo, S.W. The M2a macrophage subset may be critically involved in the fibrogenesis of endometriosis in mice. Reprod Biomed Online. 2018, 37, 254–268. doi: 10.1016/j.rbmo.2018.05.017.
38. WOESSNER, J.F. Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys. 1961, 93, 440-447. doi:10.1016/0003-9861(61)90291-0