Biochemical, radiological and histological effects of ixekizumab on skin tissue in a bleomycin-induced systemic sclerosis mouse model

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Abstract

Objective

Abnormal T-cell activation, Th17 cells, and IL-17 play a role in inflammation and fibrosis in systemic sclerosis (SSc). Targeting Th17 cells has shown promising therapeutic potential in SSc treatment. This study aimed to investigate the biochemical, radiological, and histological effects of ixekizumab (IXE), an IL-17 blocker, in a bleomycin (BLM)-induced SSc mouse model.

Methods

An experimental study was conducted on 40 female BALB/c mice, randomly divided into four groups: control, BLM, BLM+IXE, and BLM+MMF. SSc was induced by subcutaneous bleomycin administration (1 mg/kg every other day for 21 days). Following fibrosis induction, the BLM+IXE group received a single subcutaneous dose of ixekizumab (1.6 mg/kg), while the BLM+MMF group received oral mycophenolate mofetil (20 mg/kg/day) for 14 consecutive days. Skin tissue samples underwent routine histopathological processing and were stained and evaluated for various parameters using semi-quantitative scoring. Blood levels of TNF-α, TGF-β1, and IL-17A were evaluated, and skin thickness was measured via ultrasound (US). Student's t-test, Fisher's exact test, and ANOVA were used for analysis. A p-value <0.05 was considered statistically significant.

Results

TNF-α, TGF-β1, and IL-17A levels were significantly higher in the BLM group than in the control group, whereas IXE and MMF treatment partially reduced these cytokine levels. Histological analysis revealed decreased dermal fibrosis and inflammatory cell infiltration in the BLM+MMF and BLM+IXE groups compared to the BLM group. Skin thickness was greater in the BLM group on US measurements and significantly lower in the treatment groups.

Conclusion

IXE, an IL-17A inhibitor, reduced tissue fibrosis-related changes in a BLM-induced SSc model. This biological agent is thought to suppress mechanisms involved in SSc pathogenesis and showed similar antifibrotic trends to MMF, with no statistically significant differences observed between the two treatment groups for histopathological scores.

Key words

systemic sclerosis, skin fibrosis, bleomycin, ixekizumab, interleukin-17, mycophenolate mofetil, mouse model

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Introduction

Systemic sclerosis (SSc) is a chronic autoimmune inflammatory connective tissue disease characterised by widespread fibrosis of the skin and internal organs. Fibroblast activation, driven by immune activation, vasculopathy, and endothelial activation, results in increased production of the extracellular matrix (ECM) and decreased ECM degradation, which are key aspects of its pathogenesis (1, 2). Proinflammatory cytokines produced by T cells contribute to tissue fibrosis and vascular damage in SSc (2, 3). When proinflammatory cytokines such as fibroblast growth factor-1, TNF-α, and IL-1β are introduced into endothelial cell cultures, these cells can transform into myofibroblastic cells (4). Many autoantibodies, including anti-nuclear antibodies (ANA) and anti-centromere antibodies (ACA), contribute to tissue damage and inflammation in SSc (5). Abnormal T cell activation and dysregulation are observed in SSc, with CD4+ T helper cells and CD8+ cytotoxic T cells playing important roles in the pathogenesis of the disease (6).

Evidence from both SSc patients and experimental animal models indicates that IL-17 is strongly associated with fibrotic processes (7). In patients with SSc, there is evidence suggesting that Th17 cells and IL-17 contribute to the inflammation and tissue fibrosis characteristic of the disease. Th17-related cytokines are associated with disease severity and progression (8). Th17 cells can promote inflammation and fibrosis by activating fibroblasts and stimulating the production of profibrotic factors (8, 9). It has been reported that circulating Th17 lymphocytes are increased in SSc and that this increase is associated with the presence of ILD (8, 10). In a mouse model of SSc, Th17 cells participate in the pathogenesis of skin and lung fibrosis by increasing fibroblast proliferation and cytokine production (9, 11). Understanding the role of Th17 cells in SSc could lead to the development of targeted therapies aimed at modulating Th17 cell activity or IL-17 signalling, thereby reducing inflammation and fibrosis in affected tissues.

Agents such as methotrexate, azathioprine, mycophenolate mofetil (MMF), tocilizumab, and rituximab are treatment options for patients with SSc. However, many biological agents have shown limited efficacy. Understanding the roles of T cells, B cells, and cytokines in the pathogenesis of SSc is important for developing targeted therapies. IL-17 blockade may offer potential benefits in SSc management. Secukinumab, a monoclonal antibody that blocks IL-17A, has been shown to reduce dermal fibrosis in animal models (11).

Although secukinumab has been studied in animal models, the effects of ixekizumab, a higher-affinity IL-17A inhibitor, remain unexplored, representing a gap in current knowledge. Ixekizumab differs from secukinumab in its higher binding affinity to IL-17A (12). This higher affinity may enhance inhibition of IL-17A-mediated fibroblast activation and inflammatory responses, providing additional biological and therapeutic insight in SSc models. Therefore, evaluating ixekizumab may not only confirm the antifibrotic potential of IL-17A blockade but also clarify whether a higher-affinity antibody offers incremental benefits over previously studied agents.

This study aimed to evaluate the biochemical, radiological, and histological effects of IXE on skin tissue in a BLM-induced SSc mouse model (10).

Patients and methods

Study design

This experimental study was approved by Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (approval no: 64583101/2023/86). In the study, 40 female BALB/c mice (18–22 g, 6–8 weeks old) were randomly assigned to 4 equal groups (control/physiological saline, BLM, BLM+IXE, and BLM+MMF; n=10 per group, total n=40). Randomisation was performed using a computer-generated random number sequence before the experiment. The sample size (10 animals per group) was determined based on previous studies using comparable bleomycin-induced systemic sclerosis mouse models and ethical considerations for animal experimentation (13, 14). The bleomycin-induced mouse model was selected because it is

the most widely validated and reproducible experimental model of SSc, closely reproducing the key inflammatory and fibrotic features of the disease (14). The animals were housed under a 12-hour light/12-hour dark cycle with ad libitum access to food and water.

Experimental design and treatment protocol

1. Control group (n=10): received subcutaneous injections of physiological saline in volumes equivalent to those administered in the experimental groups.
2. BLM group (n=10): received bleomycin to induce SSc without subsequent therapeutic intervention.
3. BLM+IXE group (n=10): received bleomycin followed by IXE treatment.
4. BLM+MMF group (n=10): received BLM followed by MMF treatment.

To establish the SSc model, mice in the BLM, BLM+IXE, and BLM+MMF groups received subcutaneous BLM injections at a dose of 1 mg/kg in a volume of 100 μL into the dorsolumbar region every other day for 21 days. Control animals received equivalent volumes of physiological saline administered subcutaneously. Following successful induction of SSc, mice in the BLM+IXE group received a single subcutaneous injection of IXE (1.6 mg/kg, 10 μL) and mice in the BLM+MMF group were treated with MMF administered orally by gavage at a dose of 20 mg/kg once daily for 14 consecutive days.

At the end of the five-week period, dermal thickness was evaluated using ultrasound imaging. Following imaging, all animals were sacrificed under sterile conditions via cervical dislocation. For biochemical analyses, blood samples were collected via intracardiac puncture at the time of sacrifice and stored appropriately. Skin tissue samples were fixed in 4% formaldehyde for subsequent histopathological analysis (15, 16).

Histopathological assessment

Skin tissue samples were fixed in 4% formaldehyde solution and subsequently subjected to routine histopathological processing. Sections obtained from paraffin-embedded tissue blocks were stained with haematoxylin-eosin (H&E) and Masson's trichrome. Dermal thickness was assessed by measuring the distance between the epidermal-dermal junction and the dermis-subcutaneous fat boundary. In the histological evaluation, six parameters were assessed in tissue samples: fibrosis, collagen deposition, inflammatory cell infiltration, vascular damage, hair follicle damage, and sebaceous gland damage. Each parameter was scored using a semi-quantitative scale ranging from 0 to 3 based on the severity of the lesion (0: none, 1: mild, 2: moderate, 3: severe) (17, 18). All histopathological evaluations were performed by an experienced pathologist who was blinded to group allocation.

Masson's trichrome-stained histological sections were used for quantitative assessment of collagen deposition. Images were acquired at 40× magnification using an Olympus BX-50 light microscope. For each experimental group, three non-overlapping microscopic fields were randomly selected from each animal. Quantitative image analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The same threshold settings were applied to all images to ensure consistency in collagen segmentation across samples. The percentage of collagen area was calculated for each field using the following formula: Collagen area (%) = (Collagen area / Total area) × 100. For each animal, the mean value obtained from the three fields was calculated and used as the representative collagen percentage for statistical analysis.

Serum ELISA and ultrasonographic analyses

At the end of the experiment, 1 mL samples of blood were collected via intracardiac puncture from each mouse into EDTA tubes. The samples were centrifuged at 1000 × g for 15 minutes, and the serum was aliquoted into capped Eppendorf tubes. Once all samples were collected, serum levels of TNF-α, TGF-β1, and IL-17A were measured using ELISA according to the manufacturer's instructions, employing the Reed Biotech kit (RE2867H), obtained specifically for this project.

Skin thickness measurements were made in the dorso-lumbar regions of the subjects with the LA2-14A linear probe of the Samsung RS80 high-frequency ultrasonography (US) system. Imaging was conducted according to a standardised scanning protocol by a radiologist. The ultrasound system was a high-frequency linear array probe (LA2–14A) specifically designed for superficial tissue imaging. Each measurement was repeated three times per animal. The measurements were obtained from a standardised dorso-lumbar anatomical region using identical anatomical landmarks in all animals. Evaluations were conducted by a radiologist blinded to the group assignments to prevent bias. Biochemical analyses and ultrasonographic measurements were also performed by investigators blinded to group allocation, and images were obtained under identical settings for all groups. All measurements were performed by a single experienced radiologist. For reproducibility assessment, repeated measurements were used to minimise intra-observer variability, and consistency was ensured through standardised imaging parameters. For each animal, the three repeated measurements were averaged and used for statistical analysis.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) v. 25.0 (IBM Corp., Armonk, NY, USA). Numerical data were first assessed for normality using the Shapiro-Wilk test. Normally distributed variables were expressed as mean ± standard deviation (SD) and analysed using parametric tests. Non-normally distributed variables were expressed as median and interquartile range (IQR). Categorical variables were presented as frequencies and percentages (%) and analysed using Fisher's exact test or chi-square test. Histological scores, which are ordinal in nature, were analysed using the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney U-test for pairwise comparisons where appropriate. Student's t-test was used only for comparisons between two independent groups, whereas one-way ANOVA was

Table I. Histopathological scoring of skin lesions in control, BLM, BLM+MMF, and BLM+IXE groups expressed as median (interquartile range, IQR).

Parameter	Control	BLM	BLM+MMF	BLM+IXE
Fibrosis	0 (0–0)	3 (3–3)	2 (1–2)	2 (2–3)
Collagen	0 (0–0)	3 (3–3)	2 (1–2)	2.5 (2–3)
Inflammatory cell infiltration	0 (0–0)	3 (3–3)	2 (1–2)	1.5 (1–2)
Vascular damage	0 (0–0)	3 (3–3)	2 (1–2)	2 (2–2)
Hair follicle damage	0 (0–0)	3 (3–3)	2 (1–2)	2 (1–2)
Sebaceous gland damage	0 (0–0)	3 (3–3)	2 (1–2)	2 (1–2)

Table II. The distribution of serum cytokine levels in control, BLM, BLM+MMF, and BLM+IXE groups.

Serum cytokine levels	Control (mean ± SD)	BLM (mean ± SD)	BLM+MMF (mean ± SD)	BLM+IXE (mean ± SD)
TNF-α	12.1 ± 5.1	88.9 ± 32.8	15.8 ± 4.3	12.8 ± 9.5
TGF-β1	741.5 ± 437.0	6100.1 ± 2299.4	1253.3 ± 537.7	3192.0 ± 1095.4
IL-17A	3.2 ± 2.2	41.2 ± 10.0	19.4 ± 8.2	6.3 ± 3.1

used for comparisons among more than two groups. Exact *post-hoc* tests used after ANOVA have been specified. Differences in serum TNF-α, TGF-β1, and IL-17A levels among groups were evaluated using one-way ANOVA followed by Tukey's *post-hoc* test. A *p*-value below 0.05 was considered statistically significant.

Results

Histopathological assessment

In the control group, adipose tissue, subdermal adipose tissue, vascular structures, and epidermal thickness appeared normal. Fibroblast activity was low. No inflammatory cell infiltration or collagen deposition was observed.

In the BLM group, pronounced dermal fibrosis, collagen deposition, structural abnormalities in capillaries, including luminal constriction and obliteration, were observed. There was an increase in epidermal thickness and keratinisation. Inflammatory cell infiltration, primarily consisting of mononuclear cells, was prominent. Both the number and activity of fibroblasts were increased. In the subdermal adipose tissue, cell hypertrophy and focal atrophic areas were observed. There was a marked loss of hair follicles and sebaceous glands.

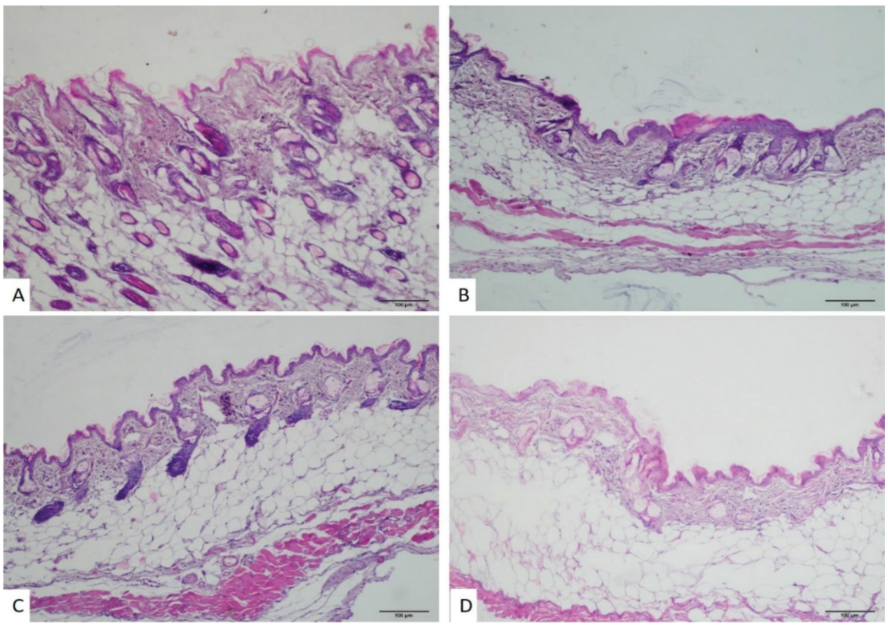

Fig. 1. Histological appearance of experimental groups (haematoxylin-eosin staining, × 20 magnification). (**A**) Control group, (**B**) BLM group, (**C**) BLM+IXE group, and (**D**) BLM+MMF group.

In the BLM+IXE group, dermal fibrosis was reduced compared to the BLM group. Collagen deposition and dermal thickness were lower than in the BLM group. Inflammatory infiltration was diminished. Endothelial damage was milder. In this experimental model, histological improvement was observed compared to the BLM group. However, the histological appearance did not return to that of the control group.

In the BLM+MMF group, dermal fibrosis was reduced compared to the BLM group. The antifibrotic effect of MMF appeared limited, and its impact on vascular inflammation was even less pronounced. Inflammatory cell infiltration was relatively decreased. Although collagen deposition was somewhat reduced, it was not as effectively suppressed as in the IXE group. Endothelial damage and vascular lesions persisted. Partial preservation of adipose tissue was observed. Overall, the observed changes suggest a partial attenuation of fibrotic and inflammatory features within the experimental context, rather than a definitive therapeutic equivalence or superiority between agents. The histological appearance of experimental groups with H&E staining and Masson trichrome staining at × 20 magnification is shown in Figures 1 and 2.

According to the histological scoring results, the BLM group showed a statistically significant increase in fibrosis, collagen, inflammatory cell infiltration, vascular damage, hair follicle damage, and sebaceous gland damage compared to the control group ($p<0.05$). Statistically significant differences were also observed between the BLM group and the BLM+IXE and BLM+MMF groups. All histopathological scoring parameters were lower in the BLM+MMF and BLM+IXE groups compared to the BLM group. However, there were no significant differences for all of these parameters between the BLM+MMF and the BLM+IXE groups (Table I). These findings indicate comparable effects on histopathological scoring parameters between the two treatment arms within the limitations of the experimental design.

Quantitative histomorphometric analysis of Masson's trichrome-stained sec-

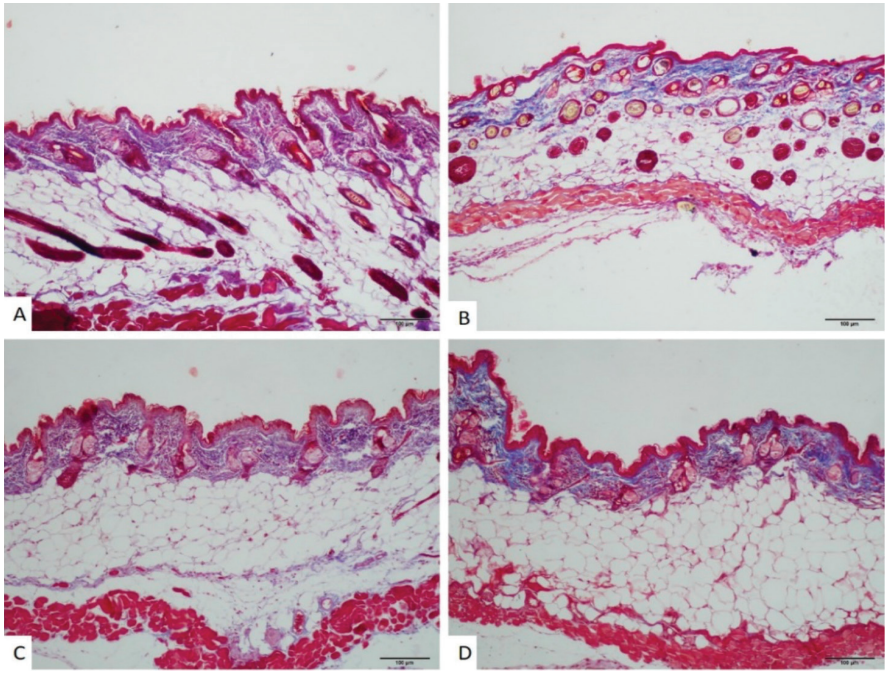

Fig. 2. Histological appearance of experimental groups (Masson's Trichrome staining, × 20 magnification). (**A**) Control group, (**B**) BLM group, (**C**) BLM+IXE group, and (**D**) BLM+MMF group.

tions revealed significant differences in collagen deposition among the experimental groups (one-way ANOVA, $p<0.05$). The BLM group exhibited the highest collagen area percentage (30.28 ± 0.28%), which was significantly greater than that of the Control group (16.20±0.06%, $p<0.0001$). Treatment with IXE significantly reduced collagen deposition compared with the BLM group (23.00±0.40% *vs*. 30.28 ± 0.28%, $p<0.0001$). Likewise, the BLM+MMF group showed a marked reduction in collagen deposition (17.54±0.40%) compared with the BLM group ($p<0.0001$). However, collagen deposition in the BLM+MMF group remained slightly but significantly higher than that in the Control group ($p=0.0007$). Furthermore, collagen deposition was significantly lower in the BLM+MMF group than in the BLM+IXE group

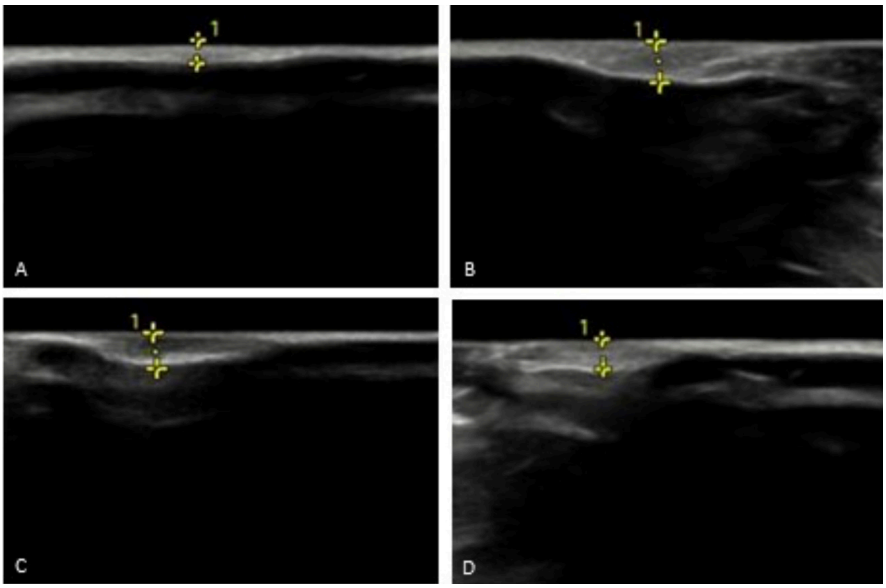

Fig. 4. Ultrasonographic images of the skin. (**A**) Control group, (**B**) BLM group, (**C**) BLM+IXE group, and (**D**) BLM+MMF group.

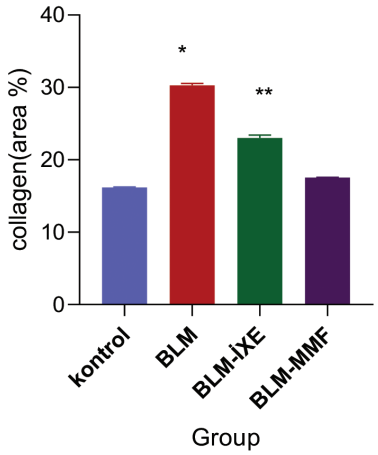

Fig. 3. Histomorphometric analysis of collagen deposition in Masson's trichrome-stained sections acquired at 40× magnification. Collagen area percentage was quantified using ImageJ.

($p<0.0001$), indicating a greater reduction in collagen accumulation with MMF treatment. Collagen deposition assessed by histomorphometric analysis of Masson's trichrome-stained sections (40×) showed group differences, as quantified by ImageJ (Fig. 3).

Evaluation of serum cytokine levels and radiological imaging

Higher levels of TNF-α, TGF-β1, and IL-17A were observed in the BLM, BLM+MMF, and BLM+IXE groups compared to the control group. When analysed quantitatively, the BLM group showed a significant increase in TNF-α, TGF-β1, and IL-17A levels compared to controls ($p<0.05$). In both treatment groups (BLM+IXE and BLM+MMF), these elevations were partially suppressed. No statistically significant difference was observed between the IXE and MMF ($p>0.05$, Tukey *post-hoc*). The distribution of cytokine levels by group is presented in Table II.

In our study, skin thickness was measured radiologically in the BLM-induced SSc mouse model. The mean skin thickness level in the control group was 1.1±0.2 mm. In the BLM group, a significantly increased skin thickness was observed compared to the other groups. Mean skin thicknesses were 2.1±0.2 mm for the BLM group, 1.7±0.4 mm for the BLM+MMF group, and 1.9±0.2 mm for the BLM+IXE group, respectively. Compared to the BLM group, IXE treatment reduced dermal thickness by

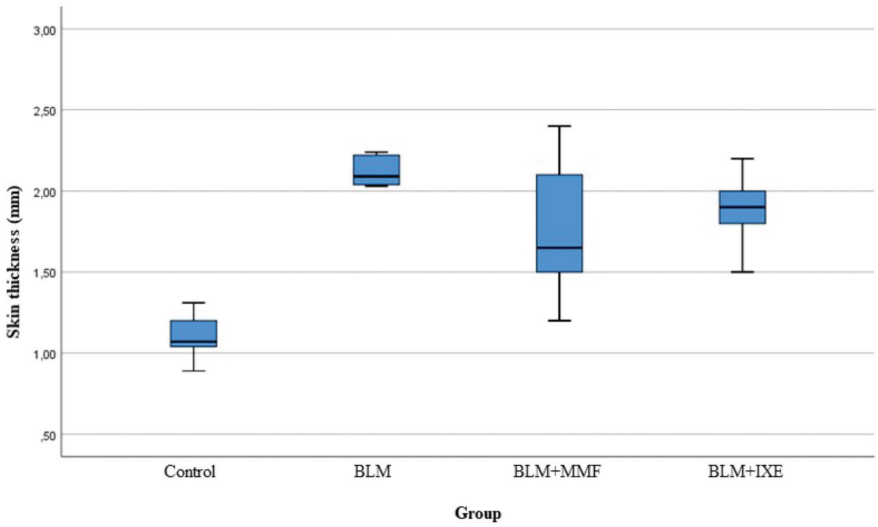

Fig. 5. The distribution of ultrasonographic skin thickness measurements. (**A**) Control group, (**B**) BLM group, (**C**) BLM+IXE group, and (**D**) BLM+MMF group.

approximately 10%, while MMF treatment reduced it by approximately 19%. No statistically significant difference was observed between BLM+IXE and BLM+MMF groups (p=0.331, Tukey *post-hoc*). US radiology images of the skin are shown in Figure 4, and the distributions of US skin thickness measurements for all groups are shown in Figure 5. Overall, these radiological findings should be interpreted as supportive experimental imaging outcomes.

Discussion

In this study, we compared the efficacy of MMF with the biological drug IXE in a BLM-induced SSc model in BALB/c female mice. The biochemical, histopathological, and radiological findings obtained in this study are consistent with established features of the BLM-induced fibrotic model. The findings primarily reflect experimental model-based fibrotic changes rather than direct clinical disease behaviour in human SSc. This model provided a useful platform for evaluating the histological, radiological, and biochemical effects of the treatments. Genetic and environmental factors and microchimerism are triggering risk factors for the endothelial cell damage and immune activation aspect of scleroderma pathogenesis (1-5). In addition to the synthesis of ECM building blocks, activated fibroblasts also produce pro-fibrotic cytokines and growth factors such as IL-6, TGF-β1, platelet-derived growth factor, and connective tissue growth factor (19). Once activated, fibroblasts acquire autocrine properties and no longer require inflammatory cell stimulation for activation. TGF-β has been identified as a regulator of pathological fibrogenesis in SSc (20). Various processes, including cell growth, apoptosis, cell differentiation, and extracellular matrix synthesis, are regulated by TGF-β, a type of cytokine secreted by macrophages and many other cell types (20). Bleomycin induces characteristic histopathological changes associated with increased lipid peroxidation, pulmonary fibrosis, and elevated inflammatory mediators (21). In our study, histopathological examinations in the BLM group revealed systemic sclerosis-like pathological findings, including dermal fibrosis, epidermal thickening, dense extracellular matrix deposition, homogenised collagen bundles, active myofibroblasts, and lymphohistiocytic inflammatory infiltration. It has been reported that a BLM-induced SSc mouse model in BALB/c female mice replicates the histopathological and immunological features of SSc (13, 14). These findings are consistent with previous studies demonstrating that BLM triggers fibrotic processes (13, 14).

In our study, although higher levels of TGF-β1 were detected in the BLM+MMF and BLM+IXE groups compared to the control group, no significant difference was found between the BLM+MMF and BLM+IXE groups. Although IXE partially reduced TGF-β1 levels, TGF-β1 remained elevated compared to controls, indicating incomplete suppression of fibrotic activity. These findings suggest that MMF and IXE reduce TGF-β1 levels and have comparable effects without statistically significant differences. This does not imply equivalence in terms of clinical efficacy and is limited to biochemical changes within the experimental model. MMF has been shown in various ways to suppress fibroblast differentiation via the TGF-β/Smad3 signalling pathway and fibrosis by ECM degradation (13, 22). In particular, MMF is associated with reduction of dermal fibrosis and significantly protects against BLM-induced skin fibrosis in a scleroderma model (13, 22). In the BLM+MMF group, a significant decrease in the collagen deposition and fibrotic changes was observed compared to the BLM group; the epidermis remained nearly normal, and inflammatory cell infiltration was significantly reduced. The decrease in TNF-α and TGF-β1 levels observed in the treatment groups in our study indicate that MMF and IXE are effective at the biochemical level against BLM-induced fibrosis.

Th17 cells are seen in the skin, subcutaneous layers, and endothelial inflammation in SSc patients (8-10). Th17 cell activity was increased and showed a positive correlation with modified Rodnan skin score, while IL-17A levels were found to be higher in patients with pulmonary arterial hypertension (23, 24). In the mouse model of BLM-induced pulmonary fibrosis, IL-17A is required for IL-1β-mediated fibrosis (11). Whether Th17 cells and IL-17A regulate the pulmonary fibrosis of SSc remains to be elucidated. Secukinumab, another monoclonal antibody that blocks IL-17A, has been shown to improve dermal fibrosis in an animal model (11). IXE blocks IL-17A, inhibiting its interaction with the IL-17 receptor expressed in various cell types, including keratinocytes. Through IL-17A inhibition, IXE is associated with reduced fibroblast activation and inflammatory cell infiltration, which may underlie its antifibrotic and anti-inflammatory

effects in SSc. Therapeutic strategies targeting M2 macrophage activity may contribute to the regulation of the profibrotic immune response in SSc (25).

Dermal fibrosis was evaluated using semi-quantitative scoring across multiple histological parameters, including fibrosis, collagen deposition, inflammatory cell infiltration, vascular damage, hair follicle damage, and sebaceous gland damage (Table I). These scores were statistically significant compared to the control group, confirming the successful establishment of the skin fibrosis model. While direct biochemical collagen measurement was not performed, the combination of histological scoring and serum cytokine analysis (TNF-α, TGF-β1, IL-17A) provides strong evidence supporting fibrosis in this model.

The quantitative histomorphometric analysis performed using ImageJ enabled an objective assessment of collagen deposition by determining the collagen-positive area relative to the total tissue area. This method has been shown to provide reliable and reproducible measurements that correlate well with conventional biochemical collagen quantification techniques, supporting its application in the evaluation of tissue fibrosis (26). In the present study, IXE treatment was associated with a significant reduction in BLM-induced collagen deposition compared with the BLM group. Although collagen accumulation was decreased, collagen levels remained significantly higher than those observed in the control group, suggesting a partial attenuation of collagen deposition under the present experimental conditions.

In our study, when IL-17A levels measured in serum were analysed, a statistically significant decrease in the IXE group was observed in addition to a decrease in TGF-β1. In the BLM+IXE group, inflammatory cell infiltration was reduced, fibroblast activation was suppressed, and the distinction between vascular injury and the BLM group remained significant. In addition, hair follicle and sebaceous gland damage was significantly reduced in both the BLM+MMF and BLM+IXE groups. IXE showed effects comparable to MMF in this experimental model, without statistically significant differences in histopathological scoring parameters between groups. This statement should be interpreted as similarity of experimental outcomes rather than therapeutic equivalence. This finding suggests that IL-17A suppression may modulate inflammation and the fibrotic process (27). These results suggest that IXE may have potential antifibrotic activity in experimental settings.

Radiologically, high-frequency US, which allows for objective assessment of skin involvement, is a non-invasive, reliable, and objective assessment tool in animal models (28, 29). In our study, skin thickness measurements obtained by US demonstrated the effectiveness of IXE treatment. In the control group, skin and subdermal fat were within normal limits, and significantly increased skin thickness was observed in the BLM group. In the BLM+IXE group, skin thickness was increased compared to the control group. This suggests that IXE may reduce fibrosis. Similarly, in the BLM+MMF group, skin thickness was greater compared to the control group but significantly lower than in the BLM group. These findings suggest that both IXE and MMF may have attenuating effects on fibrotic skin thickening in the experimental model. These imaging findings should be interpreted as supportive morphological outcomes in a murine model rather than direct evidence of clinical efficacy.

The study has several limitations, including small sample size, lack of detailed investigation into molecular mechanisms, and absence of long-term follow-up. Only serum cytokines were measured; local IL-17 expression, Th17 infiltration, TGF-β signaling, and objective collagen quantification by hydroxyproline content assay were not assessed, while collagen evaluation was based on histomorphometric analysis of Masson's trichrome-stained sections using ImageJ, limiting mechanistic conclusions. Although IXE showed beneficial effects on skin fibrosis in this preclinical model, further studies are needed to evaluate systemic organ involvement and clarify the underlying mechanisms before drawing definitive conclusions about its therapeutic potential in SSc.

To the best of our knowledge, there are no histopathological, biochemical, or radiological studies on IXE in the BLM-induced SSc mouse model. This is one of the unique aspects that our study contributes to the literature. In our study, decreases in TNF-α, TGF-β1, and IL-17A levels, as well as histological parameters and US skin thickness measurements, were observed in the IXE group. These preclinical findings suggest that IXE may be a potential therapeutic agent in fibrotic diseases such as SSc and may pave the way for further clinical studies. These preclinical findings suggest potential therapeutic promise, although systemic organ effects remain to be evaluated in future studies. However, these findings should be considered exploratory and hypothesis-generating rather than confirmatory evidence for clinical use.

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