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EARLY AND LATE HISTOPATHOLOGICAL AND RADIOLOGICAL FINDINGS OF DIFFERENT ANIMAL SPECIES AND STRAINS IN A BLEOMYCIN-INDUCED ANIMAL MODEL

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Abstract

Aim: Animal species and strains exhibit varying degrees of sensitivity to bleomycin-induced dermal fibrosis. This study aimed to establish early (week 2) and late (week 4) changes in bleomycin-induced skin fibrosis in two species (mice and rats) and three strains (BALB/C mice, C57BL/6 mice, Wistar rats) using histopathological and radiological analyses.

Material and Methods: Female C57BL/6 and BALB/C mice (n=4 each, 20-25 g, six weeks old) and female Wistar rats (n=4, 200-250 g, six weeks old) were subjected to subcutaneous bleomycin (10 mg/kg/day) or phosphate-buffered saline (PBS) every other day for four weeks. Skin biopsies from the four dorsal quadrants were analyzed for collagen homogenization, eosinophil and basophil counts, inflammatory response, and histological skin thickness (from 3 sections and ten different high-power fields in each biopsy).

Results: The collagen homogenization score, eosinophil density, mast cell density, inflammatory response score, and skin thickness were higher in the bleomycin group than in the PBS group across all models. Collagen scores were similar across models, but the inflammatory response and eosinophil and mast cell densities were higher at week 2 than at week 4. The highest inflammatory response scores at week 2 were observed in the BALB/C and Wistar mice. Histological skin thickness was greatest in Wistar rats at weeks 2 and 4.

Conclusion: The early inflammatory response was more severe in the BALB/C and Wistar models, although all models showed comparable collagen density and skin thickness. Wistar rats exhibited the most consistent parameters, making them suitable models for bleomycin-induced dermal fibrosis.

Keywords: Systemic sclerosis, animal model, mouse, rat, skin fibrosis

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INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune disorder characterized by excessive fibrosis, inflammation of the skin and internal organs, autoantibody positivity, and vascular damage (1). Fibrosis is the hallmark feature of SSc and is believed to stem from the activation and differentiation of fibroblasts into apoptosis-resistant myofibroblasts. The increased expression of myofibroblasts further catalyzes the formation of extracellular matrix (ECM), resulting in abnormal collagen deposition and pathological tissue remodeling (2). Despite considerable advancements, our understanding of the pathophysiology and key events driving persistent and uncontrolled fibroblast activation and ECM protein deposition remains incomplete (3). Consequently, there is an urgent need to elucidate the interplay between alterations in central targets to interrupt the damage cascade that precipitate disease onset. This strategy is critical for refining current therapeutic approaches for SSc, which remains one of the most devastating rheumatologic conditions to date (4).

Animal models that mimic one or more aspects of SSc have been established and are gaining recognition as valuable resources. They play a pivotal role in identifying the molecular mediators and signaling pathways involved in pathogenesis and conducting preclinical investigations to assess potential disease-modifying treatments (5,6).

Nonetheless, it is crucial to acknowledge that no single experimental model comprehensively mirrors the entire pathophysiological spectrum of human SSc. Despite their limitations, these models provide valuable insights into the mechanisms underlying unchecked fibrosis in SSc and other abnormalities associated with organ fibrosis (5,6).

The murine model of bleomycin-induced dermal fibrosis is widely used to investigate alterations occurring at various stages of the disease (7). First introduced by Yamamoto (8), the bleomycin-induced skin fibrosis model has been extensively utilized in both preclinical and pharmacological investigations. In addition to its local effects on the skin, high-dose subcutaneous bleomycin injections are believed to trigger a systemic autoimmune response characterized by the presence of autoantibodies, such as anti-nuclear autoantibodies, anti-Scl-70, and anti-U1 RNP, along with the induction of lung fibrosis. Studies have shown that bleomycin injection results in early inflammatory infiltrates comprising mononuclear cells in the skin, followed by the development of thickened dermal collagen bundles within approximately four weeks (9,10). Nonetheless, limited data are available on early and late changes in animal models generated using bleomycin across different species and strains.

Given the significance of the bleomycin-induced model of SSc in elucidating the pathogenesis of fibrotic skin changes, we investigated the early and late alterations of dermal fibrosis in the skin of two animal species, including two widely utilized mouse strains. Our hypothesis was that different species and strains manifest varying degrees of sensitivity to bleomycin-induced dermal fibrosis. The primary aim of this study was to establish methods for inducing a standardized model of fibrosis in adult rat and mouse skin. The insights gained from this investigation can serve as a reference for selecting an appropriate animal model that demonstrates morphological and histological consistency with both the early and late stages of SSc-associated skin fibrosis.

MATERIALS AND METHODS

Animals

C57BL/6 (n=4) and BALB/C (n=4) female mice weighing 20-25 g at 6-8 weeks of age and Wistar female rats weighing 200-250 g (n=4) were utilized in the study. In this study, we chose female animals to minimize the variability associated with hormonal changes that may occur in males. Literature shows that fibrosis severity can differ between sexes, and our selection aims to reduce additional sources of experimental variability. Animals were randomized into two equal groups: (I) phosphate-buffered saline (PBS)-injected and (II) bleomycin-treated groups (Figure 1). Due to known variations in bleomycin activity between batches, a unit/mL regime is generally recommended over a mg/mL regime to ensure consistent bleomycin activity. In our study, a mg/mL formulation was used, but all dosage calculations were carefully adjusted based on individual animal body weight. When changing variables such as species, age, or sex, it is important to optimize the dosage accordingly to maintain experimental reproducibility. For the early-stage SSc model, half of the animals were sacrificed at the end of the second week. The remaining animals in both groups completed the 4-week period for the late-stage SSc model. PBS and bleomycin were administered subcutaneously on weekdays (5 days) and every other day. No injections were administered on weekends (2 days). The injections were administered on the dorsal median line 1 cm superior to the tail base and 0.5 cm lateral to the right. Bleomycin (15 mg) was diluted in 15 mL of saline (1 mg/mL) and administered at a dose of 10 mg/kg (e.g., 2 mL for a 200 g rat). Similar dosages were administered to the PBS group, with 0.01 mL of PBS injected per kg. at the end of the second week, animals in the early disease arms (PBS and Bleomycin) were euthanized (n=16). The assumption that skin changes at week 2 reflect the early stage of SSc was based on previous studies (8). The dorsal region of

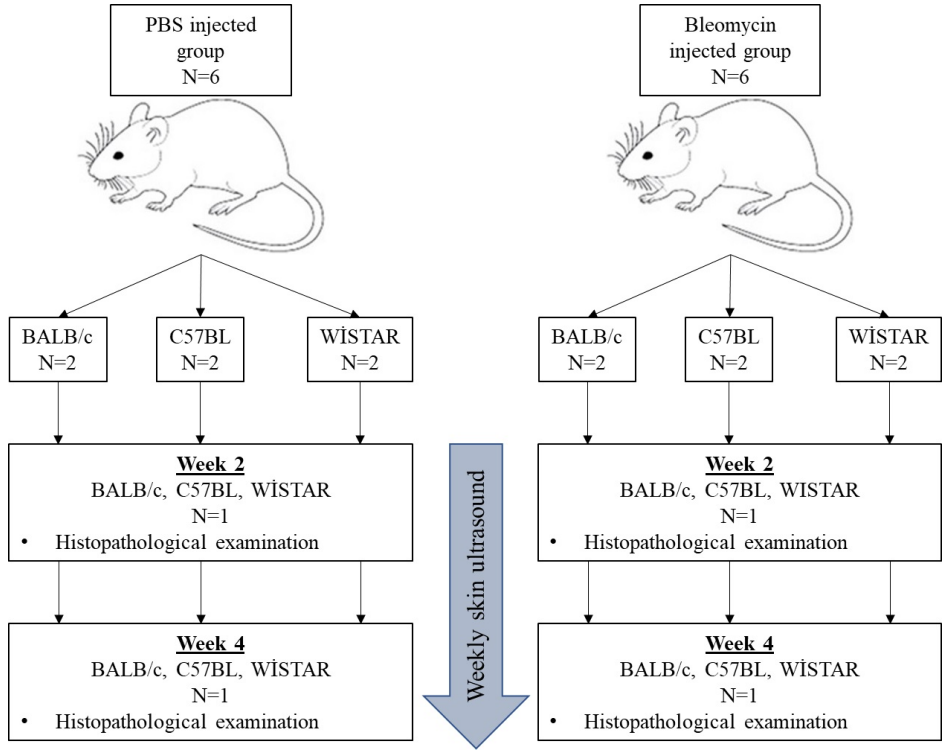


Figure 1. Schematic illustrating the procedures to be performed on the rats in the animal model
PBS: Phosphate-buffered saline

the euthanized animals was divided into four quadrants, and full-thickness skin biopsies (1x1 cm) were obtained from each quadrant for histopathological examination. The tissue samples were placed in formaldehyde for histopathological analysis. For the late-stage disease model, animals not euthanized continued to receive PBS or bleomycin injections. At the end of week 4, all animals in the late-stage disease model were euthanized. The dorsal region of euthanized animals was similarly divided into four quadrants, and full-thickness skin biopsies (1x1 cm) were obtained from each quadrant (Figure 2).

The study was approved by the Kocaeli University Animal Research Ethics Committee (approval number: KOU. Haydek2023/1, date: 31.01.2024).

Ultrasonography

High-frequency ultrasound was used to measure skin thickness weekly to assess skin thickening. This allowed us to evaluate whether the procedure-induced skin thickening and to compare skin thickness measurements between ultrasound and histopathological examination. Prior to ultrasound imaging, excess hair on the mice skin was gently shaved to prevent interference. Skin thickness was measured using ultrasound from the dorsal region of the animals (1 cm to the right and left

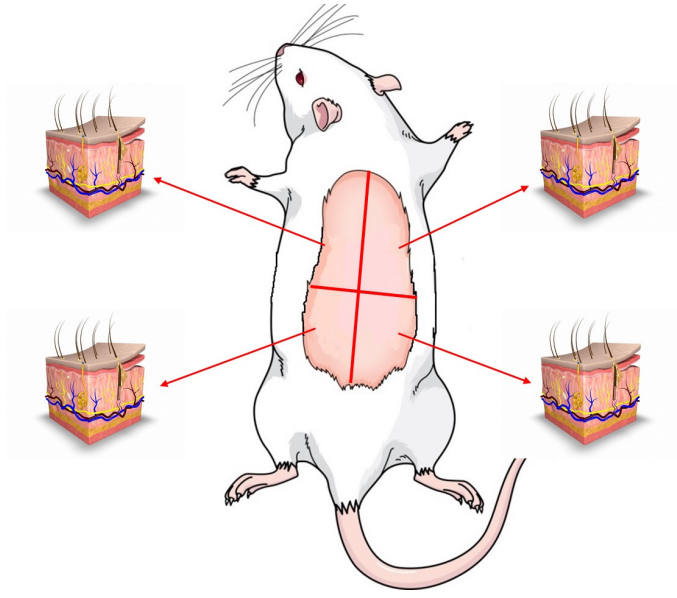


Figure 2. Schematic representation of the areas in the dorsal region of the experimental animal from which skin tissue samples will be collected

of the vertebrae, respectively) twice for each animal. Ultrasound measurements were performed longitudinally on the vertebrae using a linear probe (4-20 MHz, Esaote MyLab X9, Italy), and images were recorded (Figure 3). In the images, the epidermis-dermis thickness was manually determined by drawing a straight line, and measurements were taken from three different locations in each image, with the average being calculated.

Histopathological Examination

The collected tissues were fixed in formaldehyde for histopathological examination. Sections of skin tissue embedded in paraffin were obtained at a thickness of 4 m. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Immunohistochemical staining of tissue sections was performed according to the manufacturer's protocol. Histopathological examination was performed to evaluate skin thickness, inflammation, and fibrosis. Histological skin thickness was determined by manually drawing a straight line from the epidermis surface to subdermal adipose tissue. Skin thickness was evaluated by measuring four different sections of each biopsy sample and three different areas within each section. The results were reported as the average of the measurements. Inflammation was assessed based on the density of inflammatory cells. The number of inflammatory cells was calculated from H&E sections. The total inflammation score was based on the inflammatory response score, which was calculated using the total number of inflammatory cells in the sections. The inflammatory response score was determined by measuring four sections and three times within each section from four regions in the high-power field (x40 magnification) of each biopsy sample. The inflammatory response score was scored between 0 and 2 based on the density of all inflammatory cells (0-2=0; 3-7=1; ≥8=2). The numbers of eosinophils and basophils were similarly evaluated in four sections and

three times within each section from four regions in the high-power field (x40 magnification) of each biopsy sample from H&E sections. The degree of fibrosis density was scored from 0 to 3 based on the thickness and tightness of the collagen fibers, and the collagen homogenization score was calculated. The evaluation was performed by measuring from four sections and three times within each section from four regions in the high-power field (x40 magnification) of each biopsy sample.

RESULTS

Histopathological examination results are presented in Table 1. Collagen homogenization scores were higher in the bleomycin group than in the PBS group in both early and late stages (2 and 4 weeks) in all animals (BALB/C, C57BL/6, Wistar) (2 weeks: 1.75±0.5 vs. 0; 2 vs. 0.25±0.5; 2 vs. 0; 4 weeks: 1.75±0.5 vs. 0.5±0.58; 2 vs. 0.25±0.5; 2 vs. 0).

However, no difference was observed in collagen homogenization scores between the early and late stages of bleomycin treatment in animals.

Histological skin thickness was higher in the bleomycin-treated group than in the PBS group in all animals in the early stage (2 weeks) (1.68±0.47 vs. 0.04±0.054; 1.73±0.46 vs. 0.023±0.022; 2.25±0.51 vs. 0.068±0.066, respectively). In the late stage (4 weeks), higher histological skin thickness was observed in the bleomycin arm than in the PBS arm in C57BL/6 and Wistar strains (1.2±0.36 vs. 0.016±0.019; 3.28±0.36 vs. 0.028±0.017, respectively). Only Wistar rats reached the highest skin thickness

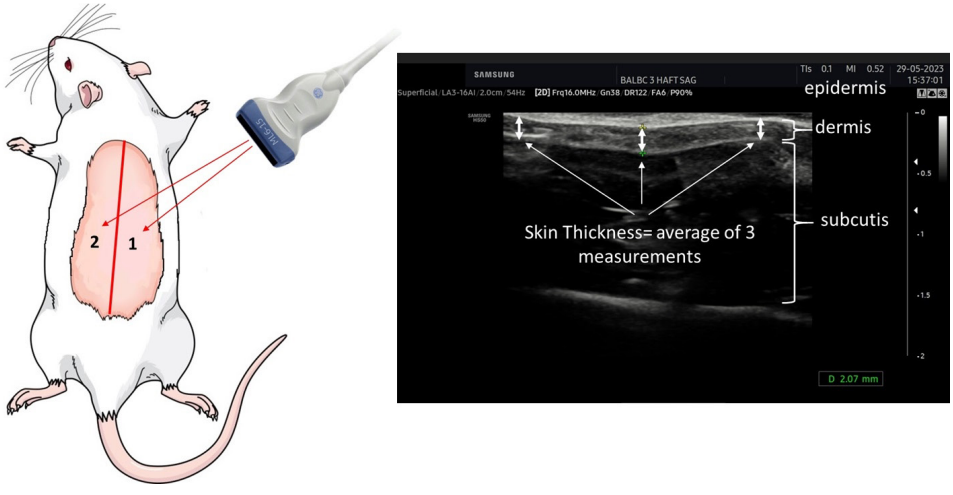


Figure 3. Schematic representation of skin measurement using ultrasound conducted in our pilot study. Along with an ultrasound image showing the measured skin tissue in a rat

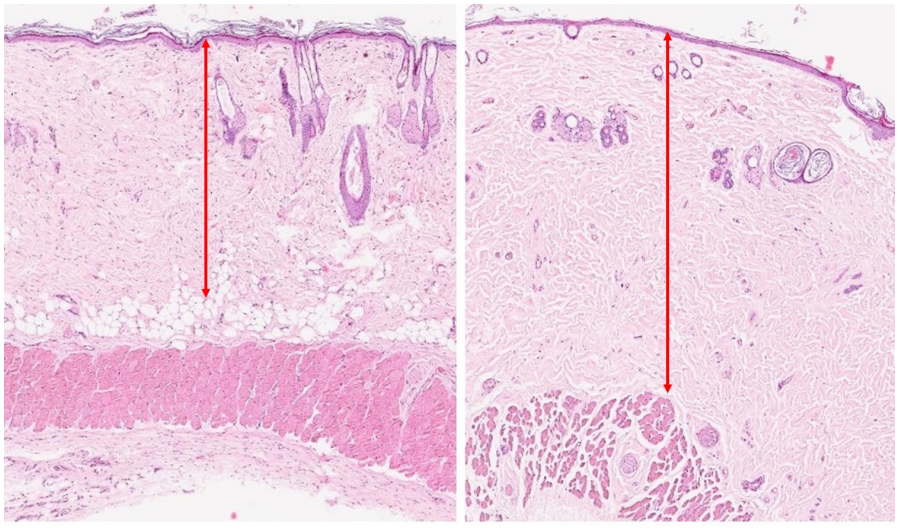
at week 4 when comparing early- and late-stage histological skin thickness in animals administered bleomycin (3.28±0.36 vs. 2.25±0.51) (Figure 4).

Inflammation, as measured by eosinophil and basophil counts and the inflammatory response score, was higher in the bleomycin group than in the PBS group in all animals at the early stage (Table 1). In the early stage (2 weeks), compared with the late stage (4 weeks), the bleomycin group showed higher eosinophil count (8.25±1.71 vs. 2.5±1.73; 3±2.58 vs. 0; 7±1.41 vs. 0), basophil count (9.75±2.76 vs. 1.25±0.96; 4.25±3.30 vs. 0; 9.5±1.29 vs. 0), and inflammatory response score (1.75±0.5 vs. 0.75±0.5; 0.75±0.5 vs. 0; 1.5±0.58 vs. 0).

Table 1. Variation in histopathological changes between the drug (BLM) and control (PBS) arms at weeks 2 and 4 based on animal species and strains

	Collagen homogenization score		Eosinophil count		Basophil count		Inflammatory response score		Histological skin thickness	
	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
BALB/C										
BLM (n=1)	1.75±0.5	1.75±0.5	8.25±1.71	2.5±1.73	9.75±2.76	1.25±0.96	1.75±0.5	0.75±0.5	1.68±0.47	1.21±1.42
PBS (n=1)	0	0.5±0.58	1.25±2.5	0	1.25±2.5	0	0.25±0.5	0	0.04±0.054	1.29±0.69
C57BL/6										
BLM (n=1)	2	2	3±2.58	0	4.25±3.30	0	0.75±0.5	0	1.73±0.46	1.2±0.36
PBS (n=1)	0.25±0.5	0.25±0.5	0.5±1	0	1±2	0	0.25±0.5	0	0.023±0.022	0.016±0.019
Wistar										
BLM (n=1)	2	2	7±1.41	0	9.5±1.29	0	1.5±0.58	0	2.25±0.51	3.28±0.36
PBS (n=1)	0	0	2.5±2.38	0	0	0	0.75±0.5	0	0.068±0.066	0.028±0.017

PBS: Phosphate-buffered saline, BLM: Bleomycin



Bleomycin week 2 Bleomycin week 4

Figure 4. Differential collagen levels in the skin fibrosis tissue induced by bleomycin were observed in the early (2nd week) and late (4th week) stages in Wistar (during the 4 week of bleomycin application, an increase in skin thickness and tightening of collagen fibrils)

Ultrasonographic measurements of skin thickness revealed higher measurements in animals treated with bleomycin than in those in the PBS group during the first weeks. From the last week onwards, skin thickness has decreased and approached that of the PBS group, except for Wistar rats. Bleomycin application significantly increased skin thickness at two weeks, followed by a decrease in the rate of skin thickness increase, reaching a plateau at week 3, and then decreasing from week four onwards (Figure 5).

DISCUSSION

Numerous murine and avian models are available to explore diverse aspects of SSc, including vasculopathy, inflammation, autoimmunity, and fibrosis. However, none of these models fully recapitulates all features of human SSc. Therefore, a rigorous selection of animal models is essential for successful *in vivo* studies. Bleomycin-induced dermal fibrosis has been established in various mouse strains, although symptom severity and the time required to induce dermal sclerosis. In this study, we aimed to investigate the changes in bleomycin-induced skin fibrosis in two different species (mice and rats) and three different animal strains (BALB/C mice, C57BL/6 mice, and Wistar rats). Additionally, we examined the differences in inflammatory and fibrotic characteristics occurring in the early (2 weeks) and late (4 weeks) stages of our model, both within each model and across the different animal models.

The bleomycin-induced skin fibrosis model offers a straightforward establishment process that can be applied to various mouse strains. This tool serves as a valuable tool for evaluating anti-inflammatory and antifibrotic therapies in preclinical studies of SSc. This model boasts advantages such as ease of implementation, widespread accessibility, and reproducibility, fulfilling essential criteria for a reliable animal model (10). The initial surge in proinflammatory cytokines [interleukin-1 (IL-1), tumor necrosis factor-alpha, IL-6, interferon-gamma] is succeeded by heightened expression of growth factors (transforming growth factor-beta 1) and extracellular components, peaking around day 14. The transition from inflammation to fibrosis typically occurs around day 9 following the initiation of bleomycin exposure. Sclerotic changes induced by bleomycin persist for at least six weeks following cessation of bleomycin injections (11,12). According to our results, compared with the control group (PBS) across all three models, we noted a substantial increase in both collagen content (collagen homogenization score) and inflammation (eosinophil count, basophil count, and inflammatory response score) at both weeks. Our findings indicated a higher level of inflammation in the BALB/C mouse model. Interestingly, we observed similar collagen homogenization scores in the early and late stages of each animal model. We interpreted this observation as potentially stemming from an increase in collagen fiber density and alterations in the molecular structure over time due to ongoing injections without resulting in discernible differences in semi-quantitative assessments.

One of the outcomes of our study revealed a notable increase in histological skin thickness compared with the control group (PBS) across all animal models during the early stage (2 weeks). However, histological skin thickness during the late stage (4 weeks) exhibited variation, with the application of bleomycin failing to induce a similar increase in the second late stage as observed in the early stage for all animals. To minimize animal sacrifice, we refrained from evaluating histological skin thickness measurements at weeks 1 and 3. Instead, we compared the radiological skin thickness measurements obtained through

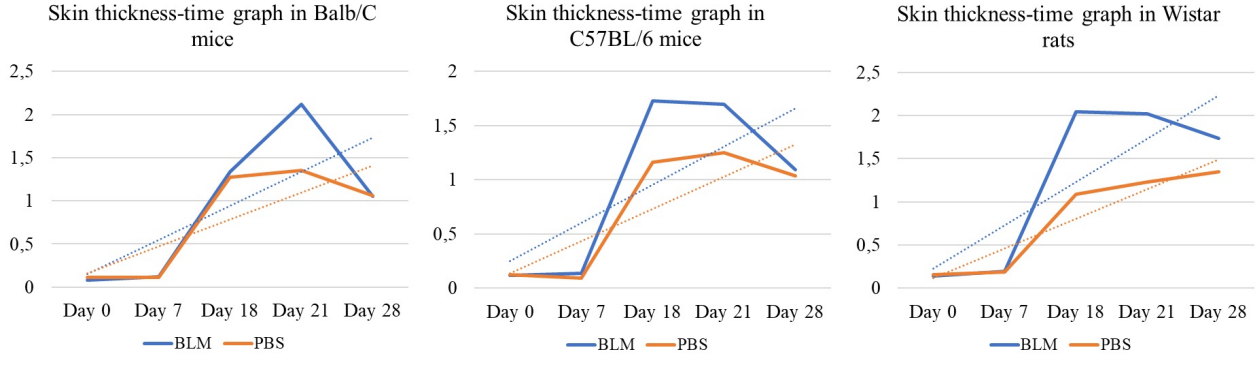


Figure 5. Variation in skin thickness over time among different animals using high-frequency ultrasound
PBS: Phosphate-buffered saline, BLM: Bleomycin

weekly high-frequency ultrasound assessments with the histological measurements. The radiological skin thickness measurements supported the observed increase in histological skin thickness, reaching a peak level at week 2, followed by a decrease in the rate of increase at week 3 and a subsequent decrease in skin thickness in all animals receiving bleomycin by week 4. The ultrasound results corroborated our histopathological findings. Histologically, we observed the thickest skin in Wistar rats during both the early and late stages. We interpreted this finding as suggestive of ongoing bleomycin application in the late stages, potentially leading to changes in collagen structure rather than only an increase in skin thickness and collagen content, which is consistent with the interpretation for collagen homogenization scores.

The number of studies that concurrently compared different species and strains in the literature is limited. In a study by Ruzehaji et al. (13), a group of male BALB/C (n=6), C57BL/6 (n=6), and DBA/2 (n=6) mice were subjected to bleomycin every other day for 21 days to investigate whether sex and mouse strain influence the severity of dermal fibrosis.

They observed the successful induction of dermal fibrosis by bleomycin in all three assessed strains, with the highest severity observed in BALB/C mice regardless of sex. Notably, female BALB/C mice exhibited greater susceptibility to bleomycin-induced dermal fibrosis compared with their female counterparts in the C57BL/6 and DBA/2 strains. Regarding dermal thickness, hydroxyproline levels, and myofibroblast counts, no significant differences were noted between male and female BALB/C and C57BL/6 mice. Male DBA/2 mice had a higher number of myofibroblasts than female DBA/2 mice. Furthermore, the inflammatory cell counts were significantly lower in male DBA/2 mice treated with bleomycin than in male BALB/C and C57BL/6 mice treated with the same. Interestingly, dermal thickness did not differ between mice administered daily bleomycin injections and those administered injections every other day.

However, compared with daily bleomycin injections, bleomycin injections administered every other day increased basal hydroxyproline levels, a biochemical marker of collagen. Moreover, there were no discernible differences in dermal thickness, hydroxyproline content, or myofibroblast counts between mice injected with bleomycin at concentrations of 0.5 mg/mL compared to those injected with 1 mg/mL bleomycin (13).

One of the most significant limitations of our study is the small number of animals. Due to the use of multiple species and strains in our study, a large number of animals was avoided. To minimize the bias resulting from this limitation, we maintained a high number of tissue samples taken from each animal and examined a high number of sections from each tissue sample. Another limitation was the use of H&E staining instead of immunohistochemical staining to identify inflammatory cells. Despite these limitations, one of the strengths of our study was that, in addition to histological examination, we simultaneously assessed the increase in skin thickness radiologically using high-frequency ultrasound.

CONCLUSION

In this study, we investigated the influence of genetic background on the induction of experimental dermal fibrosis. Our findings suggest that bleomycin injection induces dermal fibrosis in BALB/C and C57BL/6 mice as well as Wistar rats. Notably, inflammation was prominently observed in the early phase of the model and was characterized by the highest skin thickness. Increased collagen content persisted in the late phase despite a decrease in skin thickness at this stage.

Although BALB/C mice exhibit a higher level of inflammatory response than other models, Wistar rats met all the criteria for the model. These observations underscore the importance of selecting an appropriate protocol for inducing dermal fibrosis, which is relevant for pharmacological testing and therapeutic interventions. Although numerous studies in the literature have utilized animal models induced with bleomycin, concurrent assessments of different species and strains are needed. Therefore, despite the limitations of our study, our findings contribute to addressing these gaps in the literature and improve our understanding of this issue.

Footnote

Ethics Committee Approval: The study was approved by the Kocaeli University Animal Research Ethics Committee (approval number: KOU.Haydek2023/1, date: 31.01.2024.

Informed Consent: Not required.

Authorship Contributions

Surgical and Medical Practices: D.T.K., S.D.Ö., Ö.Ç., C.Ö., Concept: D.T.K., G.A., M.K., Design: D.T.K., G.A., M.K., A.Y., A.Ç., Data Collection or Processing: D.T.K., S.D.Ö., Ö.Ç., C.Ö., Analysis or Interpretation: D.T.K., S.D.Ö., Ö.Ç., A.Y., A.Ç., Literature Search: D.T.K., Writing: D.T.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

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