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Administration of Collagen Peptide Prevents the Progression of Pulmonary Fibrosis in Bleomycin-Treated Mice

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Abstract: Collagen peptides (CPs) are food-derived peptides that possess a variety of bioactive properties. Our study investigates the effects of CP on pulmonary fibrosis in bleomycin (BLM)-treated mice. C57BL/6J mice were subcutaneously injected with BLM for two weeks followed by a three-week experimental diet containing 25 mg/g of CP derived from chicken feet. Supplementation with CP suppressed the increase in lung weight and disruption of lung architecture observed in mice treated with BLM. BLM-treated mice also exhibited higher hydroxyproline content and increased expression levels of type I and III collagen subunit genes in the lungs. CP supplementation exerted no effect on these collagen-related factors; however, it significantly suppressed the gene expression of fibronectin and inflammation-related molecules in the lungs of BLM-treated mice. These findings suggest that CP administration prevents the development of pulmonary fibrosis by acting as an anti-inflammatory agent.

Keywords: bleomycin; collagen peptide; inflammatory cytokines; pulmonary fibrosis

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1. Introduction

Pulmonary fibrosis (PF) is a common pathological condition observed in numerous lung diseases and is characterized by the abnormal proliferation of fibroblasts and deposition of excessive collagen in lung tissue [1,2]. Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease of unknown etiology that is associated with high morbidity and mortality [3]. The key characteristics of IPF include the abnormal proliferation and transformation of fibroblasts, accumulation of extracellular matrix (ECM) components, and subsequent irreversible destruction of the lung architecture [4]. Inflammation plays a key role in triggering and developing fibrotic processes in the lungs; however, the precise pathogenesis of IPF is not completely understood [5]. Fibroblasts and myofibroblasts synthesize and secrete ECM, which includes collagen, proteoglycans, fibronectin, elastin, and other matrix components. In addition to suppressing inflammation, inhibiting ECM synthesis and promoting its degradation in lung tissues are promising approaches for delaying the progression of IPF [4].

To investigate the pathogenesis of PF, bleomycin (BLM)-induced animal models are widely used. BLM is an antibiotic chemotherapeutic agent used in the treatment of cancers such as lymphoma, melanoma, squamous cell cancer, germ cell cancer, and malignant pleural effusion [1]. Among the major adverse effects of this anticancer drug, fibrosis is prominent, and histological features in the lungs of BLM-treated animals resemble those observed in human IPF [1,6]. BLM administration causes inflammatory and fibrotic reactions, which result in an increase in inflammatory cytokines, followed by an increased expression of fibrotic markers [7]. A single intratracheal instillation of BLM results in peribronchial or

peribronchiolar fibrosis, whereas multiple subcutaneous injections cause fibrosis prominently in subpleural and perivascular lesions [8,9]. Subcutaneous injection has also been used as a model of scleroderma and systemic sclerosis with dermal fibrosis [10,11]. Using these animal models, several anti-fibrotic compounds have been suggested to prevent fibrosis progression [1].

Collagen peptides (CPs) are food-derived peptides that possess a variety of bioactive properties. Some of the functional peptides in CPs are absorbed into the blood as oligopeptides [12–15] and exert various bioactive effects. Many studies have reported the beneficial effects of orally administered CPs on bone metabolism, osteoarthritis, rheumatoid arthritis, and chronic joint disorders [16–20]. Furthermore, studies have indicated that CPs can also have a positive impact on metabolic syndrome-related factors, such as blood pressure [21,22], insulin sensitivity [23–25], plasma lipid levels [26], and obesity [27–29]. Although CPs exhibit a broad spectrum of physiological effects, their underlying mechanisms are not yet fully understood.

The mechanism of BLM-induced PF is known to involve cytotoxicity resulting from the excessive production of reactive oxygen species [30]. Oxidative damage and inflammatory responses can result in the activation of fibroblasts and the subsequent development of fibrosis in the lungs [31,32]. Various studies have evidenced that CPs exert anti-inflammatory effects and suppress the secretion of inflammatory cytokines [1,25,33,34]. Additionally, CPs possess antioxidant activity [33–36] and fibroblast proliferation-modulating effects [37,38]. Nevertheless, no studies have addressed the effectiveness of CPs in the treatment of inflammation and fibrosis in fibrotic diseases, such as IPF. In this study, we investigated whether the administration of CPs affects pulmonary inflammation and subsequent PF in BLM-treated mice.

2. Results

2.1. Body Weight and Food Intake

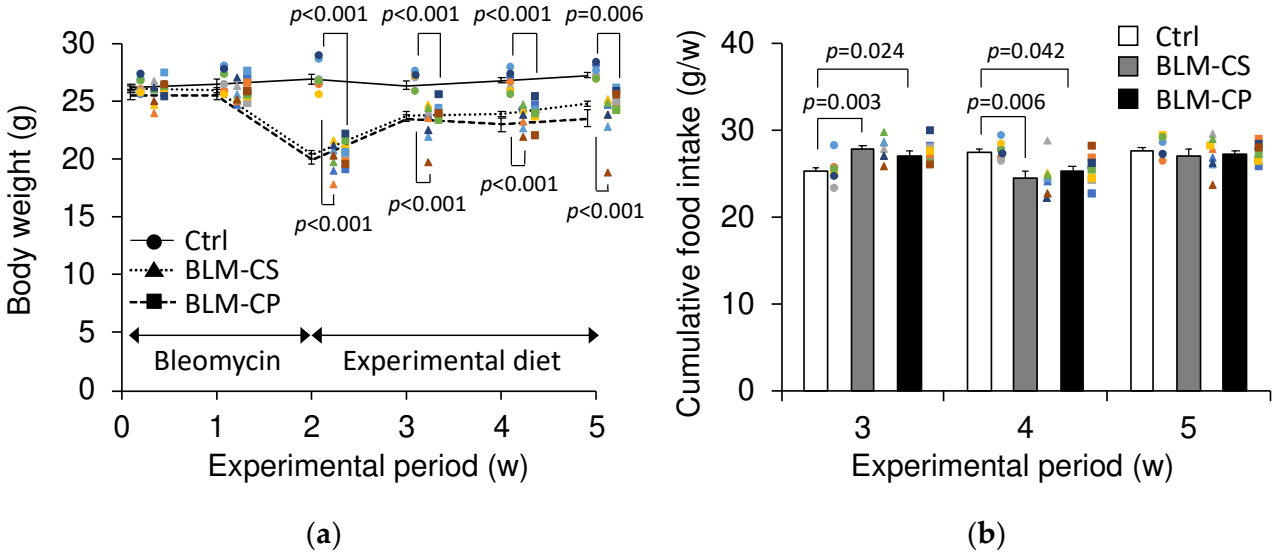
In this study, C57BL/6J mice were subcutaneously injected with BLM for two weeks and then each fed an experimental diet for three weeks. Figure 1a shows the change in body weight of the mice in the Ctrl (saline-treated), BLM-CS (BLM-treated and casein-administered), and BLM-CP (BLM-treated and CP-administered) groups during the BLM injection period and the subsequent experimental diet-feeding period. Body weight loss post-BLM treatment was partially recovered during the experimental diet period; however, the final body weights of the mice in both BLM-treated groups (BLM-CS and BLM-CP) were significantly lower than those in Ctrl group ($p < 0.01$). These results suggest that BLM treatment in C57BL/6J mice resulted in persistent body weight loss, which is widely seen in patients with IPF [39].

Figure 1b shows the cumulative weekly food intake of the mice in each group during the experimental diet period. At weeks 3 and 4, the cumulative food intake of the mice in both BLM-treated groups was significantly higher and lower, respectively, than that in the control group, whereas no significant difference was observed at week 5. During the experimental diet period, there was no significant difference in cumulative weekly food intake between the BLM-CS and BLM-CP groups. The average amount of CP ingested by the BLM-CP group was 95 mg/day.

2.2. Weight of Organs

Table 1 shows the final body and organ weights of the mice in each group at the end of the experimental period. There were no significant differences observed in the weights of the heart, liver, and spleen among the three groups. The kidney weights in both BLM-treated groups were significantly lower than those in Ctrl group ($p < 0.001$); however, there were no significant differences in the relative weights (mg/g body weight). In contrast, the lung weights in the BLM-treated groups were significantly higher than in the Ctrl group ($p < 0.001$). Interestingly, the mice in the BLM-CP group showed significantly decreased lung weight compared to those in the BLM-CS group ($p = 0.020$). These results indicate that

BLM treatment increased lung weight in mice, and this increase could be suppressed via CP supplementation.



(**a**) (**b**)

Figure 1. Body weight changes (**a**) and cumulative food intake (**b**) of mice in each group during the experimental period. (**a**) Body weight changes during bleomycin (BLM)-injection period (1–2 weeks) and experimental-diet-feeding period (3–5 weeks). Values obtained from 7 (Ctrl group) or 8 (BLM-CS and BLM-CP groups) mice are shown as mean ± SEM. Small circles (Ctrl), triangles (BLM-CS), and squares (BLM-CP) indicate individual values. The initial (0) week of the experimental period corresponds to 9 weeks of age. (**b**) Cumulative weekly food intake during experimental-diet-feeding period. Bars indicate mean ± SEM of the mice in each group. Small circles, triangles, and squares indicate individual values. Note: during BLM injection period, all animals were provided a commercial pelleted diet.

Table 1. Final body and organ weights of the mice in each group.

		Ctrl	BLM-CS	BLM-CP
Body weight	(g)	27.2 ± 0.22 [a]	23.6 ± 0.74 [b]	24.8 ± 0.25 [b]
Heart	(g)	0.125 ± 0.003	0.119 ± 0.004	0.117 ± 0.003
	(mg/g BW)	4.58 ± 0.126	5.12 ± 0.329	4.73 ± 0.133
Kidney	(g)	0.370 ± 0.010 [a]	0.317 ± 0.006 [b]	0.304 ± 0.007 [b]
	(mg/g BW)	13.6 ± 0.38	13.6 ± 0.62	12.3 ± 0.30
Liver	(g)	1.14 ± 0.04	1.08 ± 0.04	1.13 ± 0.03
	(mg/g BW)	41.7 ± 1.35	46.3 ± 2.02	45.6 ± 0.89
Lung	(g)	0.176 ± 0.007 [a]	0.294 ± 0.015 [b]	0.250 ± 0.007 [c]
	(mg/g BW)	6.48 ± 0.26 [a]	12.7 ± 1.07 [b]	10.1 ± 0.33 [c]
Spleen	(g)	0.089 ± 0.005	0.081 ± 0.006	0.086 ± 0.002
	(mg/g BW)	3.28 ± 0.198	3.44 ± 0.255	3.48 ± 0.092

Values obtained from 7 (Ctrl group) or 8 (BLM-CS and BLM-CP) mice are shown as the mean ± SEM. BW, body weight. [a,b,c] Mean values indicated by dissimilar letters are significantly different ($p < 0.05$).

2.3. Hydroxyproline Content in Lung Tissue

Collagen content in the lung tissues was evaluated via the quantification of hydroxyproline, a collagen-specific amino acid. Figure 2 shows the hydroxyproline content in the lung tissues of mice in each group. Mice in the BLM-CS and BLM-CP groups showed significantly higher hydroxyproline content than those in the Ctrl group ($p < 0.05$). No significant differences were observed between the BLM-CS and BLM-CP groups.

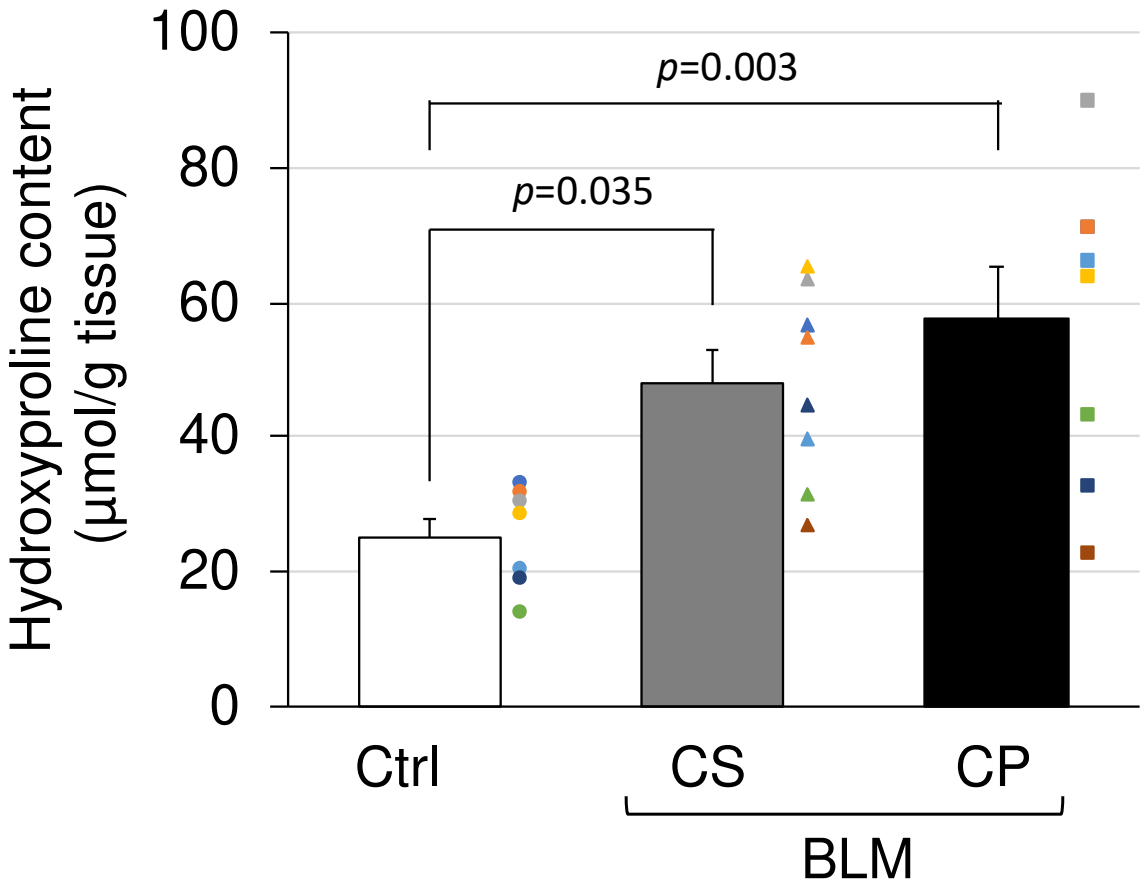


Figure 2. Hydroxyproline content in lung tissue. Bars indicate mean ± SEM of the mice in each group. Small circles, triangles, and squares indicate individual values.

2.4. Expression Levels of Fibrosis-Related Genes in Lung Tissue

The gene expression of several factors involved in ECM synthesis in the lungs was measured using a real-time polymerase chain reaction (PCR). Figure 3 shows the expression levels of type I and III collagen subunits (*Col1a1* and *Col3a1*), fibronectin (*Fn1*), and cellular communication network factor 2 (*Ccn2*, also known as connective tissue growth factor, CTGF) in the lung tissues of mice in each group. In both BLM-treated groups, the expression levels of *Col1a1*, *Col3a1*, and *Fn1* were significantly higher than those in the Ctrl group ($p < 0.01$). No significant differences were observed between BLM-CS and BLM-CP groups regarding the expression levels of *Col1a1* and *Col3a1*. In contrast, *Fn1* expression in the BLM-CP group was significantly lower than that in the BLM-CS group ($p = 0.001$). The expression level of *Ccn2* was moderately increased in the BLM-CS group ($p = 0.053$ versus the Ctrl group), and the level in the BLM-CP group decreased to a level comparable to that observed in the Ctrl group. These results suggest that CP administration decreases the synthesis of some ECM components, but does not affect the expression levels of genes involved in collagen production.

2.5. Expression Levels of Inflammatory Cytokine Genes in Lung Tissue

The gene expression levels of inflammatory cytokines in the lung tissues of the mice were determined. As shown in Figure 4, the expression levels of *Il6* (encoding interleukin (IL)-6) and *Ccl2* (encoding monocyte chemoattractant protein-1, MCP-1) were significantly higher in the BLM-CS group than in the Ctrl group ($p < 0.001$). The expression levels of both genes were significantly lower in the BLM-CP group than in the BLM-CS group ($p < 0.05$). Additionally, the expression levels of *Il1b* (encoding for IL-1β) and *Tgfb1* (encoding for transforming growth factor (TGF)-β1) genes were significantly lower in the BLM-CP group

than in the BLM-CS group ($p < 0.05$). These results indicate that CP administration may suppress lung inflammation by inhibiting the increase in pro-inflammatory cytokines.

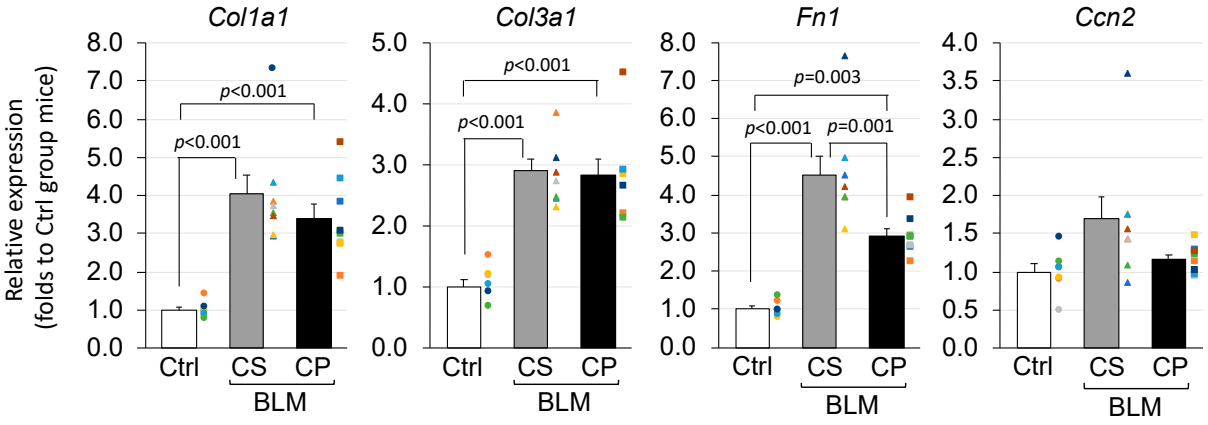


Figure 3. Expression levels of fibrosis-related genes in lung tissue. Gene expression levels in lung tissues were determined using real-time PCR. Values are represented as the fold change for each BLM group compared to the Ctrl group. Bars indicate mean ± SEM of the mice in each group. Small circles, triangles, and squares indicate individual values.

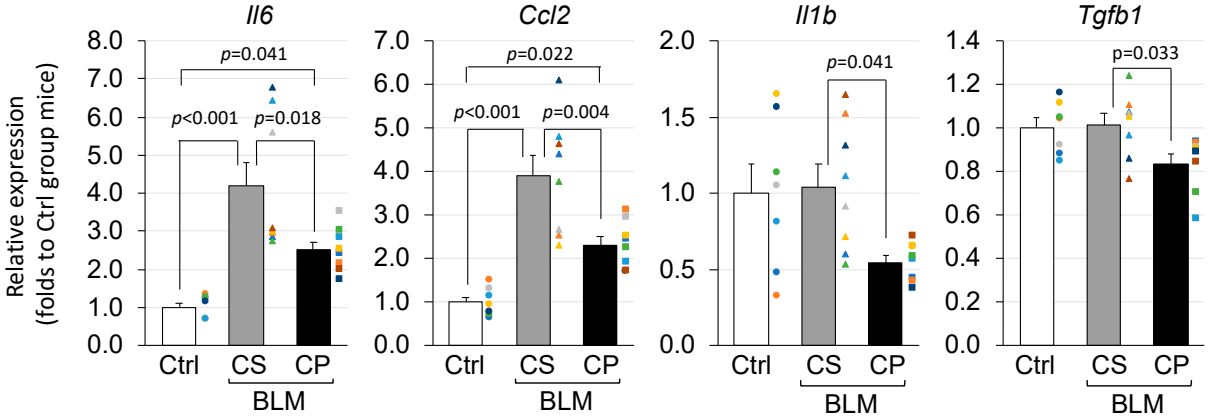


Figure 4. Expression levels of inflammatory cytokine genes in lung tissue. Gene expression levels in lung tissues were determined using real-time PCR. Values are represented as the fold change for each BLM group compared to the Ctrl group. Bars indicate mean ± SEM of the mice in each group. Small circles, triangles, and squares indicate individual values.

2.6. Histological Analysis of Lung Tissue

Figure 5a shows hematoxylin and eosin (H&E) staining of mouse lung sections. The lung sections obtained from mice in the Ctrl group showed a normal histological structure of the alveoli, which comprised thin interalveolar septa and clear alveolar sacs. In contrast, mice in the BLM-CS group showed disruption of the lung architecture, including thickening of the interalveolar septa, cellular infiltration, and decreased alveoli. Mice in the BLM-CP group showed improved lung architecture and decreased cellular infiltration. The lungs of mice in both the BLM-CS and BLM-CP groups exhibited subpleural fibrosis with pleural thickening and the deposition of collagen fibers, which were stained with picrosirius red (Figure 5b). These results suggest that CP administration can prevent the disruption of lung architecture following BLM exposure.

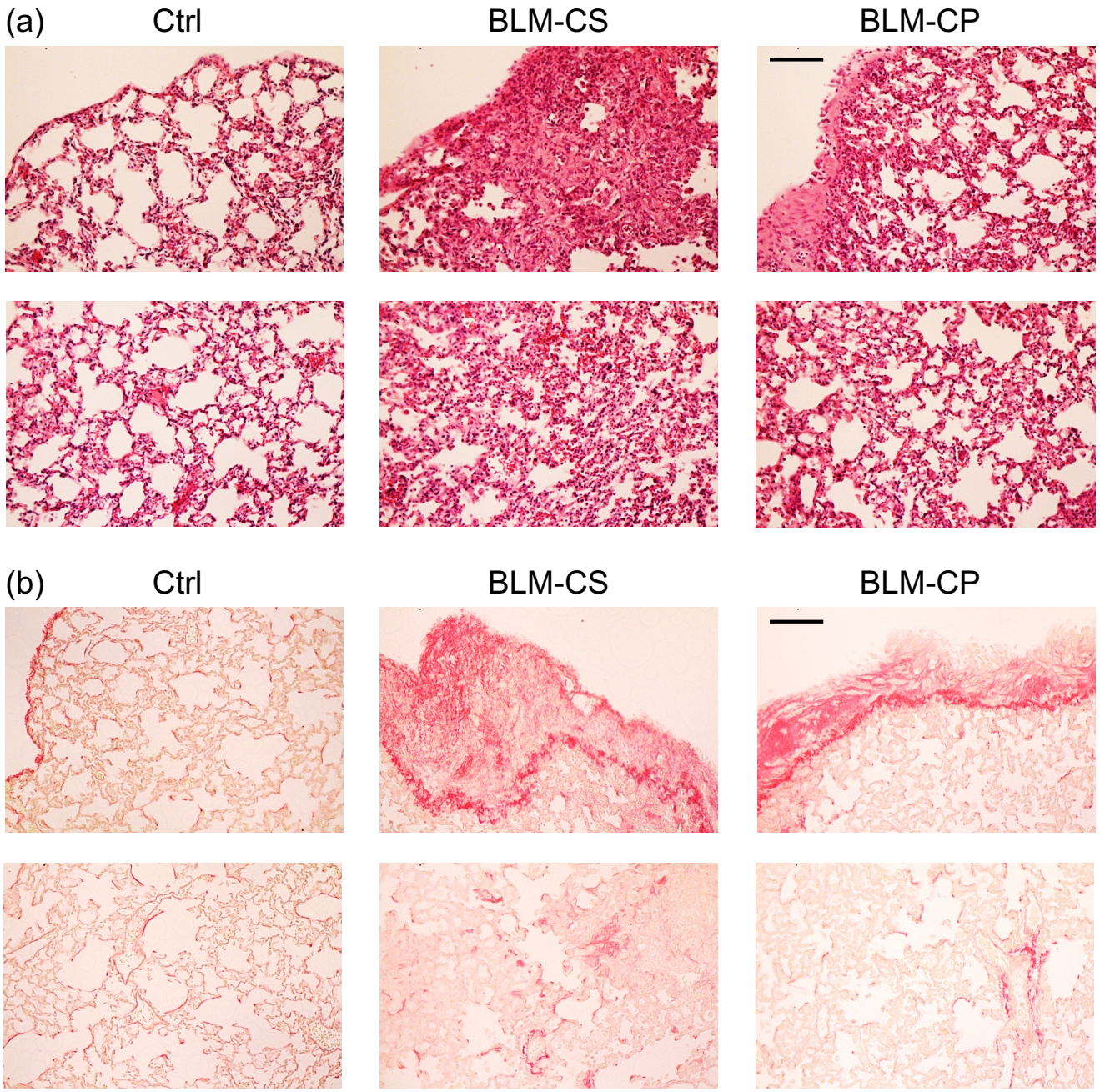


Figure 5. Histological structures of lung tissue. (**a**) Hematoxylin and eosin (H&E) staining of paraffin-embedded lung tissue sections obtained from the mice in each group. (**b**) Picrosirius red staining of the sections. Representative images from each group are shown. Scale bars correspond to 100 μm.

3. Discussion

The present study showed that the administration of chicken-feet-derived CP suppressed the progression of PF in BLM-treated mice. The cytotoxicity of BLM is known to depend on the expression of BLM hydrolase, a BLM-inactivating enzyme. As the lungs and skin lack this enzyme, they are most susceptible to BLM-induced tissue injury [1,40]. In this study, multiple subcutaneous infusions of BLM significantly increased lung weight in mice but not in other internal organs (Table 1). Histological analysis of lung tissue sections demonstrated that BLM-treated mice exhibited disrupted lung architecture, including cellular infiltration and decreased alveoli (Figure 5). The administration of CP suppressed lung weight increase and improved lung architecture disruption in BLM-treated mice,

suggesting the prevention of interstitial pneumonia and the progression of fibrosis. The average amount of CP ingested by the BLM-CP group (95 mg/day) was similar to that reported in our previous studies [19,28]. The quantity of supplemental CP protein in the experimental diet was equivalent to 10% of the total protein content, which reflects 5 g of CPs in humans who ingest 50 g of protein per day.

Mice in the BLM-treated group had higher levels of hydroxyproline in their lungs, reflecting increased collagen content. However, unlike in lung weight, no significant difference was observed between the BLM-CS and BLM-CP groups (Figure 2). Consistently, there were no significant differences in the expression levels of the collagen fiber subunit genes (*Col1a1* and *Col3a1*) between the BLM-CS and BLM-CP groups, although these levels were significantly higher than those in the Ctrl group (Figure 3). These observations suggest that the administration of CP suppressed the progression of BLM-induced lung fibrosis but did not inhibit collagen fiber synthesis.

In contrast, CP administration significantly decreased the expression of fibronectin, a fibril constituent. Additionally, the CP treatment suppressed the gene expression of inflammation-related molecules in the lungs of BLM-treated mice. The progression of fibrosis involves a variety of profibrotic cytokines produced by injured cells, fibroblasts, and immune cells, such as macrophages. IL-6 and MCP-1 are representative inflammatory cytokines implicated in the pathogenesis of various fibrotic diseases via the promotion of inflammation. The expression levels of these genes were increased four-fold by BLM treatment, whereas they were decreased by half with CP supplementation. Furthermore, CP administration reduced the expression of IL-1β and TGF-β1, which are profibrotic cytokines that play crucial roles in regulating PF [41]. The mice in the BLM-CP group exhibited a lower expression of these genes than the mice in the Ctrl group, although the differences were not significant ($p = 0.078$ for *Il1b* and $p = 0.066$ for *Tgfb1*). Since the mice in the Ctrl group were injected with the same volume of saline solution as those in the BLM-treated groups, it is possible that CP supplementation reduced inflammatory cytokines in mice independent of BLM-induced oxidative damage. These findings suggest that CP may prevent the development of interstitial pneumonia and fibrosis progression through its anti-inflammatory action, rather than by directly inhibiting the fibrotic process.

The chicken-feet-derived CP used in this study mainly consisted of tripeptides and a considerable amount of longer oligopeptides, including nonapeptides and hexapeptides [13]. It has been reported that various oligopeptides isolated from chicken-cartilage-derived CPs exhibited an anti-inflammatory effect through inhibiting the secretion of inflammatory cytokines such as IL-1β and TNF-α [33]. The present study did not identify the bioactive components of CP, and further investigation is required to elucidate the mechanisms underlying the anti-inflammatory effects of CP in BLM-treated PF mice. Additionally, casein was used as a reference protein for CP supplementation in this study. Because CPs contain a high proportion of glycine, proline, and hydroxyproline, and their amino acid compositions are largely different from those of casein, it is crucial to confirm the effectiveness of CP administration in treating PF compared with the same composition of amino acid mixtures.

In this experiment, CP was administered to mice for three weeks following BLM injection. As the production of proinflammatory cytokines precedes the enhancement of profibrotic markers, compounds used during the early phase of BLM-induced lung injury act as anti-inflammatory agents [1,42]. To determine whether CP has true antifibrotic potential, it is necessary to evaluate the efficacy of CP administration in the late fibrotic stage of the experimental model. Additionally, the mice in this study received multiple subcutaneous injections of BLM to induce PF. As daily subcutaneous injections of BLM exhibit a variable distribution of lesions, including dermal fibrosis, this administration route is commonly used as a model for scleroderma and systemic sclerosis [10,11]. Accordingly, it is possible that generalized inflammatory conditions affected the progression of lung fibrosis in this animal model. Future approaches using an IPF model with a single

intratracheal instillation of BLM would be helpful to elucidate the inhibitory effect of CPs on the development of PF.

4. Materials and Methods

4.1. Animals and Experimental Diet

Eight-week-old male C57BL/6JJmsSlc mice were obtained from Japan SLC Inc. (Shizuoka, Japan). The mice were housed individually in an air-conditioned room maintained at 23 °C and 40–50% humidity under a 12 h light–dark cycle. CP (C-LAP, a low-molecular-weight collagen hydrolysate derived from chicken feet) was purchased from Nipponham Food Ltd. (Osaka, Japan). The amino acid composition of C-LAP has been reported previously [13]. Casein was purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). The experimental diets for the groups were prepared using a commercial powdered diet (CE-2-powder, CLEA Japan Inc., Tokyo, Japan). To prepare the experimental diet, 25 mg of casein (for Ctrl and BLM-CS groups) or CP (for BLM-CP group) was added to 1 g of powdered CE-2 and 2 mL of water, as previously described [28].

4.2. Experimental Design

All experiments were performed using a single set of mice. Twenty-five mice were fed a commercial pelleted diet (CE-2-pellet) for one week. BLM (Nippon Kayaku, Tokyo, Japan; 2.5 mg/mL saline solution) was administered to 18 mice at a dose of 10 μg/g body weight via subcutaneous injection five times per week for two weeks. Seven mice were assigned to the Ctrl group and were administered the same volume of saline. After administration of BLM, two mice that exhibited severe body weight loss and reduced activity were humanely euthanized and excluded from the analysis. The BLM-treated mice were then divided into two groups: BLM-CS and BLM-CP. These groups were fed the respective experimental diets, as described in the previous section, for a period of three weeks with unrestricted access. The daily amount of food consumed by each mouse was monitored, and the body weight of each mouse was measured three times per week. At the end of the experimental period, blood samples were collected from the mice under anesthesia in the morning following a 12 h fast. The plasma was separated from the blood cells immediately by centrifugation and stored at −80 °C until further analysis. The liver, kidney, spleen, heart, and lung tissues were excised and weighed. Tissues from right lung were immediately stored at −80 °C for subsequent RNA isolation and the determination of hydroxyproline content.

4.3. Histological Analysis of Lung Tissue

A portion of left lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin blocks. The paraffin-embedded tissues were sectioned into 5 μm thick slices and stained with H&E following standard deparaffinization and rehydration procedures. For the visualization of collagen fibers, sections were stained using the picrosirius red method [43]. For evaluating the severity of fibrosis, Ashcroft scores were assessed using a scale ranging 0 (normal lung) to 8 (total fibrous obliteration of fields) in H&E-stained sections [44].

4.4. Determination of Hydroxyproline Content in Lung Tissue

The total hydroxyproline content in the right lung was measured using a commercial Hydroxyproline Microplate Assay Kit (Bioworld Technology, Inc., Bloomington, MN, USA). Briefly, tissue samples (0.05 g) were transferred into screw-capped glass tubes and 1 mL of 6 M HCl was added. Then, the samples were heated at 110 °C for 8 h and centrifuged at 12,500× *g* at 25 °C for 20 min. The hydroxyproline content of the supernatant was measured according to the manufacturer's protocol and expressed as micrograms of hydroxyproline per 1 g wet tissue.

4.5. RNA Isolation and cDNA Preparation from Lung Tissue

Total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cells were suspended and homogenized in 1 mL TRIzol®, and total RNA was isolated according to the manufacturer's protocol. cDNA was synthesized from total RNA using the PrimeScript® RT Master Mix (Takara Bio Inc., Shiga, Japan).

4.6. Quantitative Analysis of Gene Expression by Real-Time PCR

The primers used in this study are listed in Table 2. The relative amounts of transcripts in the cDNA samples were determined via real-time PCR using TB Green Premix Ex Taq™ II, Tli RNaseH Plus (Takara Bio), and PicoReal 96 real-time PCR system (Thermo Fisher Scientific). The PCR was performed at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C, and the relative gene expression was analyzed using the delta-delta Ct method. The specificity of the amplification was confirmed via melting curve analysis. The relative amounts of the transcripts were normalized to those of β-actin (*Actb*) in the same cDNA.

Table 2. Primers used in this study.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Accession Number
Actb	CTTGGGTATGGAATCCTGTGG	GTACTTGCGCTCAGGAGGAG	NM_007393
Ccl2	GTCTGTGCTGACCCCAAGAA	TGCTTGAGGTGGTTGTGGAA	NM_011333
Ccn2	AGCAGCTGGGAGAACTGTGT	GCTGCTTTGGAAGGACTCAC	NM_010217
Col1a1	TGACTGGAAGAGCGGAGAGT	GAATCCATCGGTCATGCTCT	NM_007742
Col3a1	TGGTCCTCCAGGAGAAAATG	GACCAGGAGAACCAGAAGCA	NM_009930
Fn1	TGAGCGAGGAGGGAGATGAA	TAGGTGCCTGGGGTCTACTC	NM_010233
Il1b	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	NM_008361
Il6	CGGCCTTCCCTACTTCACAA	CAAGTGCATCATCGTTGTTCA	NM_031168
Tgfb1	GTCACTGGAGTTGTACGGCA	TCATGTCATGGATGGTGCCC	NM_011577

Actb, β-actin; *Ccl2*, chemokine (C-C motif) ligand 2; *Ccn2*, cellular communication network factor 2; *Col1a1*, collagen type I α1; *Col3a1*, collagen type III α1; *Fn1*, fibronectin 1; *Il1b*, interleukin 1β; *Il6*, interleukin 6; *Tgfb1*, transforming growth factor, β1.

4.7. Statistical Analysis

Statistical significance was evaluated using analysis of variance (ANOVA), and multiple comparisons were performed using the Tukey–Kramer test. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using R software (version 4.1.3, the R Project for Statistical Computing, Vienna, Austria).

5. Conclusions

In conclusion, the results of this study suggest that CP supplementation prevents the development of PF by acting as an anti-inflammatory agent. This is a novel function of CPs, which are shown to have various health benefits as food-derived bioactive peptides.

Fibrosis is not only associated with PF but is also implicated in various collagen-related diseases and age-related organ deterioration. However, the BLM-induced PF model, which involves inflammatory responses linked to acute oxidative lung injury, has limitations in understanding the chronic, irreversible progression of IPF's etiology [1,9]. Therefore, it is important to confirm whether CPs prevent the progression of lung fibrosis in different animal models. It is anticipated that the efficacy of CPs in fibrosis will be demonstrated in future clinical trials.

Author Contributions: Conceptualization, S.K.; Methodology, S.K., M.Y. and H.S.; Validation, S.K. and M.Y.; Formal Analysis, S.K. and M.Y.; Investigation, M.Y., C.A., A.M. and K.I.; Resources, S.K.; Data Curation, S.K. and M.Y.; Writing—Original Draft Preparation, S.K. and M.Y.; Writing—Review and Editing, S.K., Y.S.-M. and H.S.; Visualization, S.K. and M.Y. Supervision, S.K., Y.S.-M. and H.S.; Project Administration, S.K.; Funding Acquisition, S.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal experiments were approved by the Animal Ethics Committee of the University of Niigata Prefecture (approval number 2202, June 2022) and conducted in accordance with the institutional guidelines for the Care and Use of Animals of the University of Niigata Prefecture, following the National Research Council's Guide for the Care and Use of Laboratory Animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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