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ORIGINAL ARTICLE

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Gelatinases Increase in Bleomycin-induced Systemic Sclerosis Mouse Model

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ABSTRACT

Systemic sclerosis is a fibrotic autoimmune disease in which aberrant remodeling of the extracellular matrix in organs disturbs their functionalities. The aim of this study was to investigate the expression of gelatinases on systemic sclerosis. Consequently, a mouse model of systemic sclerosis was employed and the gelatinolytic activity of gelatinases was evaluated on the fibrotic tissues of this model.

Two groups of ten mice were considered in this work: a group of systemic sclerosis model and control group. For the generation of systemic sclerosis model, mice received bleomycin, while the control group was subjected to phosphate buffered saline (PBS) reception. Mice were tested for fibrosis by using trichrome staining, hydroxyproline measurement and α -SMA detection in tissue sections. Additionally, the gelatinolytic activity of matrix metalloproteinase 2 and matrix metalloproteinase 9 were measured using gelatin zymography in lungs and skin tissue homogenates.

The obtained results indicated that subcutaneous injection of bleomycin-induced fibrosis in skin and lung tissues of mice. Pro and active forms of matrix metalloproteinase 9 were increased in fibrotic lung tissues ($p < 0.05$ and $p < 0.01$, respectively), while, the gelatinolytic activity of MMP2 was unaffected in these tissues. Additionally, in skin tissues of bleomycin-treated animals, both pro and active forms of MMP9 and MMP2 were increased ($p < 0.05$).

Pro and active forms of gelatinases increase differently in skin and lung tissues of bleomycin-induced scleroderma.

Keywords: Bleomycin; Gelatinase; Matrix metalloproteinase 2; Matrix metalloproteinase 9; Systemic scleroderma

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INTRODUCTION

Systemic sclerosis (SSc) is a fatal multisystem autoimmune disease in which tissue homeostasis is disrupted. Dysregulation of the immune system and production of auto antibodies cause vascular endothelial and alveolar epithelial cell injury; consequently, the fibrotic process is initiated in tissues. Cellular injury and aberrant healing lead to accumulation of extracellular matrix (ECM) proteins, especially collagen, in different organs.¹ Excessive deposition of ECM results in tissue fibrosis which compromises tissue architecture and organ functions.² The amount of ECM proteins in tissues is controlled through the balance between their production and degradation by matrix metalloproteinases (MMPs).²

Matrix metalloproteinases are a family of 25 zinc-dependent proteinases which are capable of degrading all extracellular matrix components. Most of MMPs are secreted as inactive zymogens and activated by proteolytic remodeling.³ At steady state, these proteins are not constitutively expressed; however, the exogenous stimuli such as various cytokines, growth factors, inflammation and cell to cell contact can induce their expression.^{4,5} Additionally, due to central roles of MMPs in morphogenesis, wound healing, tissue repair and remodeling, their activity is rigorously regulated.^{6,7} Dysregulation of MMP activities may lead to various pathological diseases such as inflammation, arthritis, cancer, and fibrosis.⁸⁻¹⁰

MMP2 (gelatinase A) and MMP9 (gelatinase B), gelatinases, are the two well-known enzymes from MMPs family. It is generally accepted that MMP2 and MMP9 have considerable impacts on the degradation of fibrillar collagens after their initial cleavage by collagenases.^{3,11} Also Investigations demonstrate that these two gelatinases have direct collagenolytic activities and the ability to cleave several other ECM proteins such as elastin, type IV collagen, and latent TGF- β .³ Accordingly, gelatinases have an important function in ECM homeostasis and pathogenesis of fibrotic diseases. In an animal model of idiopathic pulmonary fibrosis (IPF) the activity of gelatinases increases in lung tissues. It is indicated that the type of gelatinases and their expressing cells are different in the early and late phase of disease episodes.^{12,13} These studies proposed that MMP2 and MMP9 are involved in the establishment of fibrosis in the early phase of

disease but in late phase, they may be involved in tissue repair process.^{12,13}

Animal models are inevitable tools for understanding the pathogenesis of human disease. To the best of our knowledge, no study has investigated the alterations of gelatinases in tissues of an animal model of systemic sclerosis. Due to the immense impact of gelatinases in fibrotic disease, we induced a mouse model of systemic sclerosis with bleomycin (BLM) and evaluate the gelatinolytic activity of gelatinases in fibrotic murine tissues. Subcutaneous injection of bleomycin for 4 weeks described by Yamamoto et al, induces a mouse model of scleroderma.¹⁴ In this model, skin fibrosis is accompanied by lung fibrosis, inflammation, and production of auto antibodies, which closely resemble human SSc.¹⁴

MATERIALS AND METHODS

Animal Model of Systemic Sclerosis

In order to investigate the activity of gelatinases in systemic sclerosis (SSc), an animal model of disease was employed. Six to eight weeks old female BALB/c mice were acquired from Pasteur Institute (Tehran, Iran) and were kept at the Center of Experimental and Comparative Studies (Tehran, Iran). The mice were kept in the standard laboratory cages at room temperature and ambient humidity under a 12-hour reverse light-dark cycles. They received ad libitum access to the normal laboratory chow and water. Prior to the beginning of the experiments, the mice were retained in the laboratory cages for a period of two weeks in order to acclimate to the laboratory conditions. Then, they were randomly divided into two groups of 10 mice termed as Bleomycin (BLM) and control (Phosphate buffered saline (PBS)) groups. SSc was induced in mice according to protocol suggested by Yamamoto et al.¹⁴ Briefly, each mouse in BLM group received daily 0.15 unites of bleomycin which was dissolved in 100 μ L PBS. Bleomycin (Nippon Kayaku, Tokyo, Japan) was injected subcutaneously into the shaved back skins of mice daily for a period of 28 days. In this approach control mice (PBS) received 100 μ L PBS subcutaneously for 28 days. It should be noted that all of the conducted animal experiments in the current research are approved by the Animal Care Committee of Iran University of Medical Sciences (N.: IR.IUMS.FMD.REC1394.25723).

Tissue Harvesting, Histology, and Immunohistochemistry

On day 29 under deep anesthesia, the chest was opened and lungs were removed. The skin in bleomycin or PBS injection site was excised. The left lungs and half of the excised injured skins were fixed overnight in 10% neutral formalin. Paraffin-embedded 4 μm tissue sections from lung and skin were stained with Hematoxylin-eosin and Masson's trichrome.

In microscopic examination, fibrosis was detected by the tissue content of collagen which was stained blue by trichrome staining. The dermis thickness was defined as the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction.¹⁵ After image acquisition from trichrome sections under the light microscope at 100X magnification, the thickness was measured by ImageJ software (Fiji-Win64) in 5 different skin field of each specimen.¹⁵ Tissue microscopic evaluations were performed by two independent observers.

The appearance of myofibroblasts is an essential step in tissue repair and fibrosis formation process. Alpha-smooth muscle actin (α -SMA) is a useful marker for the identification of myofibroblasts. For detection of α -SMA, 5 μm sections from injured skin and lung were deparaffinized and rehydrated. After the antigen was retrieved with fresh sodium citrate buffer, sections were blocked with H₂O₂ and bovine serum albumin. Slides were incubated with conjugated primary antibody α -SMA-HRP (1:500, Santa Cruz Biotechnology, USA) overnight at 4°C. The chromogen diaminobenzidine tetra hydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) was added until sufficient color developed. Finally, sections were counterstained with hematoxylin. For quantification, α -SMA positive cells were counted in all microscopic fields of skin tissue sections. In lung tissues, percent of α -SMA positive areas were calculated by imageJ software in images of 10 random microscopic fields, then the mean percent of α -SMA positive areas was determined for each specimen.

Hydroxyproline Assay

Collagen deposition was estimated as a marker for fibrosis by hydroxyproline measurement. After tissue excision, two upper lobes from right lungs and the one-fourth of the excised skins were immediately snap frozen and stored at -80°C until the process of hydroxyproline assay. Hydroxyproline was measured

with hydroxyproline assay kit (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer protocol.

Gelatin Zymography

Gelatin zymography was performed according to the Toth and Fridman protocol to detect semi quantitatively pro and active levels of MMP2 and MMP9.¹⁶ MMP2 and MMP9, due to their gelatinolytic activity, hydrolyse gelatin, that was embedded in the polyacrylamide gel in the gelatin zymographic method. After coomassie staining, areas of degradation are visible as clear bands against a darkly blue stained background.

For gelatin zymography, lower lobes of right lungs and other one-fourth of excised skins were homogenized in ice-cold homogenization buffer (25 mM Tris-HCl PH 7.5, 100 mM NaCl, 1% Triton X-100 and Protease inhibitor) and centrifuged at 10,000 \times g for 10 min at +4°C. The supernatants were stored at -40°C until further analysis.

Total protein concentrations were measured with the bicinchoninic acid assay (Pars Tous, Iran). For each sample, 20 μg of total protein was mixed with non-reducing sample buffer and electrophoretically (constant voltage 125) separated in SDS polyacrylamide (10%) gel containing 1 mg/ml gelatin. Gels were then treated with 2.5% Triton X-100 for 45 min at room temperature to remove SDS. Zymograms were subsequently incubated 16 hours at 37 °C in developing buffer (50 mM Tris-HCl, pH 7.8, 0.2 M NaCl, 5 mM CaCl₂, and 0.02% v/v Triton -X 100). The gels were stained with 0.5% Coomassie Blue R-250 and destained in 10% acetic acid and 30% ethanol in dH₂O. Human recombinant MMP9 (R&D) protein was used as positive control. Prestained protein marker (SMOBIO 9-245 kDa) was used as molecular weight standard. For densitometric analysis of bands, imageJ software was used.

Statistics

Statistical analysis was performed using Graph Pad Prism (version 8). Unpaired t-test was used for data analysis. All data are expressed as mean \pm SEM. Differences were considered significant when probability value (p)<0.05 (denoted as *), p <0.01 (denoted as **), p <0.001 (denoted as ***) and p <0.0001 (denoted as ****).

RESULTS

Bleomycin-induces Fibrosis in Skin and Lung Tissues of Mice

Bleomycin injection caused fibrosis in skin and lung tissues of mice as we described in our previous work.¹⁵ Fibrosis was accompanied with an increase in

dermis thickness and hydroxyproline amount in skin and lung tissues of mice.¹⁵

Bleomycin injection enhanced the expression of α -SMA in skin and lung tissues of mice compared with control group ($p < 0.05$ in the skin and $p < 0.001$ in the lung) (Figure 1A, B, C).

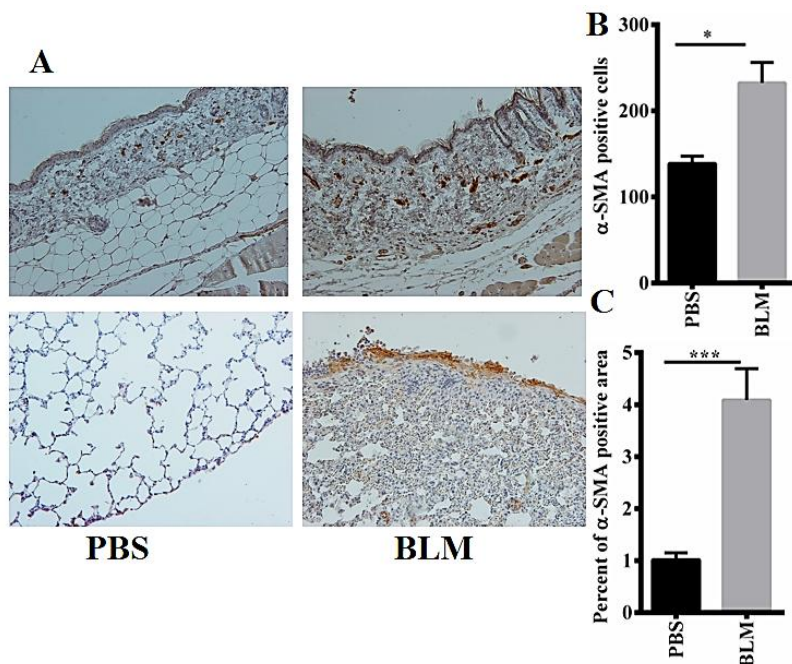


Figure 1. Bleomycin increases the expression of α -SMA in skin and lung tissues of bleomycin-induced systemic sclerosis mouse model. (A) Immunohistochemistry staining of α -SMA in skin and lung tissues of control group and bleomycin-induced systemic sclerosis mouse model group. Original magnification was 100X for skin (top) and 200X for lung slides (below). (B) Quantitative expression of α -SMA in skin tissues of control group and bleomycin-induced systemic sclerosis mouse model group. (C) Quantitative expression of α -SMA in lung tissues of control group and bleomycin-induced systemic sclerosis mouse model group. Results are presented as Mean \pm SEM. N=9-10 mice in each group. Data was analysed with unpaired t-test. BLM=Bleomycin group, PBS=Control group, α -SMA=Alpha-smooth muscle actin, p =probability value, *= $p < 0.001$, *= $p < 0.05$**

MMP9 Increases in Bleomycin-induced Lung Fibrosis

Zymographic analysis from lung tissue lysates of control group showed little or no bands of pro MMP9 and pro MMP2. Bleomycin administration significantly induced the expression of MMP9 bands in zymograms (Figure 2A). Administration of bleomycin increased the expression of active and pro MMP2 in a non-significant manner ($p > 0.05$) (Figure 2B). The mean expression of active MMP9 in the bleomycin-treated group was 2.9 fold higher than the control group (26503 \pm 5658 vs

9121 \pm 1850, $p < 0.01$) (Figure 2C). Additionally, the expression of pro MMP9 in BLM group was significantly higher than the control group (16660 \pm 5405 vs 4476 \pm 1058, $p < 0.05$) (Figure 2C).

MMP9 and MMP2 Increase in Bleomycin-induced Skin Fibrosis

Analysis of gelatin zymographic gels demonstrated that MMP2 was the main gelatinase in skin tissues. In most normal skin lysates, MMP9 did not show any activity (Figure 3A). Bleomycin-induced-fibrosis

augmented gelatinase activity in zymograms (Figure 3A). Expression of pro and active forms of MMP2 in skin zymograms of bleomycin received mice was intensified compared with control group (respectively 4411 ± 758.8 vs 1813 ± 192.0 , 3213 ± 488.2 vs

1632 ± 224.5 , $p < 0.05$) (Figure 3B). Pro and active MMP9 increased in skin tissues of bleomycin received mice compared with the control group (respectively 29043 ± 9779 vs 6044 ± 1813 , 18438 ± 6016 vs 4590 ± 1084 , $p < 0.05$) (Figure 3C).

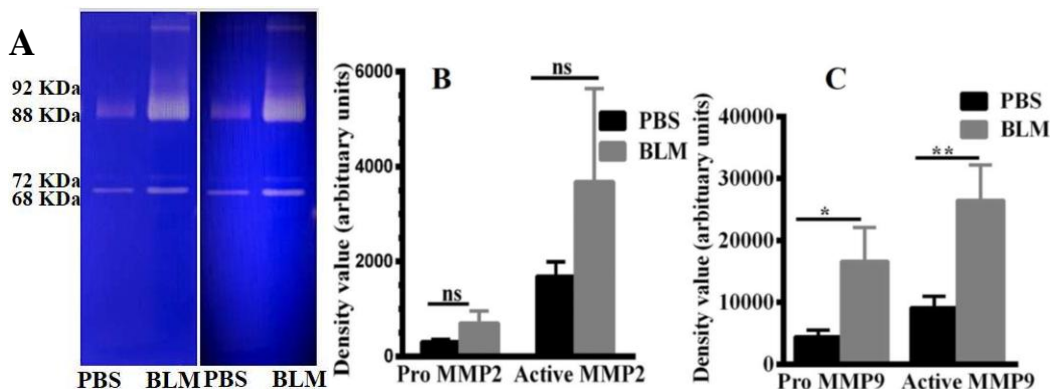


Figure 2. MMP9 increases in lung tissues of bleomycin-induced systemic sclerosis mouse model. (A) Gelatin zymography of lung tissue lysate. 92 KDa=Pro MMP9, 88 KDa=Active MMP9, 72 KDa=Pro MMP2, 68 KDa=Active MMP2. (B) Density values of MMP9 in lung tissues. (C) Density values of MMP2 in lung tissues. Results are presented as Mean±SEM. N=9-10 mice in each group. Data was analysed with unpaired t-test. BLM=Bleomycin group, PBS=Control group, p =probability value, $ns=p > 0.05$, $**=p < 0.01$, $*=p < 0.05$

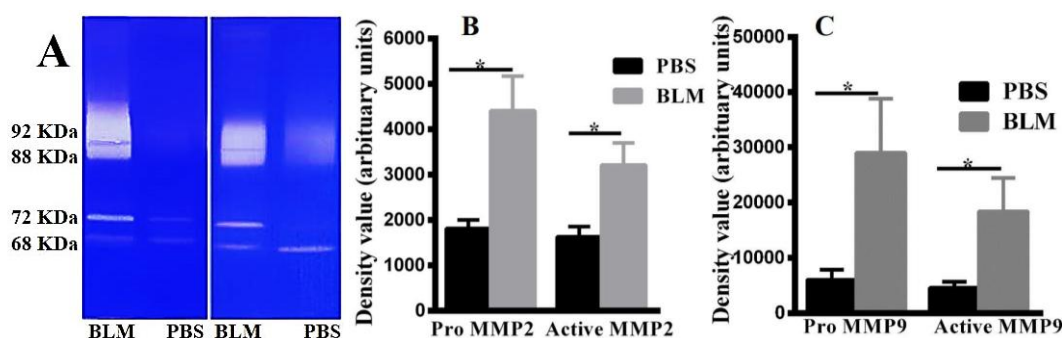


Figure 3. Gelatinases increase in skin tissues of bleomycin-induced systemic sclerosis mouse model. (A) Gelatin zymography of skin tissue lysate. 92 KDa=Pro MMP9, 88 KDa=Active MMP9, 72 KDa=Pro MMP2, 68 KDa=Active MMP2. (B) Density values of MMP2 in skin tissues. (C) Density values of MMP9 in skin tissues. Results are presented as Mean±SEM. N=9-10 mice in each group. Data was analysed using unpaired t-test. BLM=Bleomycin group, PBS=Control group, p =probability value, $*=p < 0.05$

DISCUSSION

The obtained experimental results demonstrate that gelatinases increase in the lungs and skin tissues of a

mouse model of systemic sclerosis.

Systemic sclerosis (SSC) is a multisystem fibrotic autoimmune disease. Cell injury and aberrantly sustained production of cytokines, growth factors, and

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angiogenic factors in SSc result in a deregulated tissue repair. These deregulated repairs in a tissue tilt its homeostasis toward hyperplasia and excessive accumulation of extracellular matrix (ECM).¹ Excessive deposition of ECM components in tissues of an organ leads to fibrosis which destroys the operations of the organ.¹ Due to the importance of ECM in the maintenance of organ function, regulation of its homeostasis is strictly critical. Studies emphasize that MMPs and their endogenous inhibitors represent a fundamental effect in this maintenance and their alterations can play a crucial role in the pathogenesis of fibrotic diseases.² Additionally, MMPs have a significant task in immunity and repair particularly in the procedures like cell migration, leukocyte activation, antimicrobial defense, and chemokine processing.¹⁷ As stated previously, the general function of MMPs is the degradation of the ECM proteins. It is argued that this functionality diminishes in fibrotic organs; however, considering the other operations of MMPs, this notion may be inaccurate.¹⁸ Accordingly, animal models and in vitro studies indicate pro and anti-fibrotic effects for MMPs. MMP3 and MMP7 in the mouse model of idiopathic pulmonary fibrosis (IPF) show pro-fibrotic function. Overproduction of MMP3 leads to fibrosis, as a result, bleomycin-induced fibrosis was dramatically reduced in MMP3^{-/-} and MMP7^{-/-} mice.^{19,20}

Over expression of MMP2 and MMP9 was confirmed in IPF patients, their expressions were seen in myofibroblasts, neutrophils, and epithelial cells.^{21,22} Also, alveolar macrophages of these patients expressed excessive amounts of MMP9 which was reduced by immunosuppressive therapy.²³ The increment of gelatinases causes the disruption of the basement membrane and the invasion of fibroblast to the alveolar spaces which in turn benefits the fibrotic process.^{21,22} In SSc patients with interstitial lung disease (SSc-ILD) amount and activity of MMP9 is increased in bronchoalveolar lavages compared with bronchoalveolar lavages of SSc patients without ILD and healthy controls.²⁴ Even though, there are no data available concerning the expression of these two gelatinases in tissues of SSc-ILD patients. Our results showed overexpression of pro and active form of MMP9 in fibrotic lung tissue and these are in accordance to results obtained from bronchoalveolar lavages of SSc-ILD patients and also compatible with results obtained from IPF patients.

In bleomycin-induced IPF, the expression of MMP9 is elevated especially in the early phase of disease induction. In this phase, MMP9 contributes to disruption of the alveolar epithelial cell basement membrane and progression of fibrosis. In the late stages, the gelatinolytic activities of the latent and active forms of MMP2 are prominent, and MMP2 is localized to the regenerated alveolar epithelial cells. MMP2, especially its active form, possibly plays a role in alveolar epithelial cell regeneration.^{12,13} In our study MMP9 was elevated but the increment of MMP2 was not seen, this event may be due to the use of different delivery protocols of bleomycin. In IPF model direct instillation of bleomycin occurred one time through trachea but in subcutaneous injection delivery of bleomycin occurs for 28 days period.²⁵ In our model, chronic injury exists and repair does not occur until the injection is stopped, therefore increment of MMP2 for repairing was not seen in this model. Necessarily, further investigation would be done to evaluate the gelatinase activity after the induction of fibrosis and with the initiation of repair in a mouse model of scleroderma.

Despite the assumed function of MMP9 in lung fibrosis, the MMP9 null mice developed fibrosis similar to that of wild-type after bleomycin administration.²⁶ By contrast, over expression of MMP9 in transgenic mice decreased bleomycin-induced fibrosis.²⁷ The discrepancy between the results reported by different groups can be partly due to the timing of pathological assessment. Fibrosis process initiates with injury, inflammation, repair and finally resolution, the latter being was disrupted in the fibrotic disease. It is assumed that if MMPs are expressed in the early phase of disease induction, they have a pro-fibrotic effect but in late stages, they have an anti-fibrotic effect.¹⁸ Our study was done in the late stage of disease, therefore overexpression of MMP9 in fibrotic lung tissues may have an anti-fibrotic and protective effect against the bleomycin-induced fibrosis.

MMP2 is expressed constitutively by several types of skin cells, but the expression of MMP9 is restricted in normal skin tissues.²⁸ Myofibroblasts from SSc patients and healthy controls do not express MMP9 but the activity of MMP2 is enhanced in SSc fibroblasts.²⁹ The serum level of MMP2 in scleroderma patients is not different from normal controls, while the serum level of MMP9 is elevated in SSc patients. Skin scores in SSc patients are positively correlated with the serum

level of MMP9.^{30,31} In contrast to lung tissue (IPF patient), the expression of MMP9 in the skin of diffuse cutaneous SSc patients are low, but this expression does not alter in limited cutaneous disease.³² In keeping with results of other studies, our results indicate that in all samples of control skin, the gelatinolytic activity of MMP2 exists but the amount of MMP9 is low in normal skin tissues. We detected the increment of MMP2 in fibrotic skins but our obtained MMP9 data was different from results achieved from SSc patients, this may be due to the different origin of samples. However, it is obscure whether the overexpression of MMPs is a defense reaction of the body against the fibrogenic effect of bleomycin or this overproduction has synergy with the fibrogenic effect of bleomycin. There is still much unknown concerning the action of gelatinases in SSc based on the complexity in their functions, regulation and the lack of animal studies. Therefore further animal studies are required.

In this study, we could not evaluate the activity of gelatinases and their inhibitors (TIMPs) in fibrotic tissues before and after the disease induction. Also, it is necessary to mention that gelatinase zymography is a semiquantitative method therefore further techniques such as ELISA are required to reveal the real level of gelatinases.¹⁶

In conclusion, according to other studies, our study shows that MMPs activity is different and specific in various organs. The major gelatinase in normal lung tissue is MMP-9 while in skin tissue the MMP2 is highly expressed. In fibrotic lung and skin tissues, amount of gelatinases increased differently.

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All animal works were done according to approval Number IR.IUMS.FMD.REC1394.25723.

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