A Master's Dissertation

TMF and Catechol attenuate pulmonary fibrosis by inhibiting ROS-mediated EGFR phosphorylation and EMT

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4′,6,7-트리메톡시이소플라본과 카테콜이 활성산소 매개 표피성장인자수용체의 인산화와 상피-간엽 전이를 저해함으로써 폐섬유화에 갖는 효능 연구

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TMF and Catechol attenuate pulmonary fibrosis by inhibiting ROS-mediated EGFR phosphorylation and EMT

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A thesis submitted in partial fulfillment of the requirement for the degree of Master in Medicine

2022. 07.

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LIST OF ABBREVIATIONS

α-SMA	Alpha smooth muscle actin
BALF	Bronchoalveolar lavage fluid
BLM	Bleomycin
COL4A6	Collagen type 4 α 6
DPI	Diphenyleneiodonium chloride
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IPF	Idiopathic pulmonary fibrosis
LYM	Lymphocyte
MAPK	Mitogen-activated protein kinase
NAC	N-Acetyl Cysteine
NEU	Neutrophil
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
pEGFR	Phospho-epidermal growth factor receptor
pERK	Phospho-extracellular-signal-regulated kinase
pSMAD2	Phospho-Smad2
ROS	Reactive oxygen species
TGF-β	Transforming growth factor-β
TMF	4',6,7-trimethoxyisoflavone

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PART 1.

ROS affects EGFR phosphorylation, resulting in an acceleration of pulmonary fibrosis - *in vitro* analysis

Ⅰ. ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive and dysregulated response to alveolar injury which results in excess extracellular matrix (ECM) production. Associated with high mortality, IPF is generally incurable by current pharmacological therapies.

However, the molecular pathway through which IPF occurs remains unclear. Uncovering the molecular mechanism will contribute to developing effective antifibrotic treatment strategies.

This study focused on the role of Epidermal Growth Factor Receptor (EGFR) in accelerating fibrosis. I investigated the molecular axis behind Reactive Oxygen Species (ROS) and the phosphorylation of EGFR, treating human alveolar epithelial cells (A549) with EGF and transforming growth factor (TGF)-β1. This *in vitro* approach suggested that increased activation of EGFR, as a result of its phosphorylation supported by ROS, can accelerate fibrosis.

By scavenging ROS or inhibiting NADPH Oxidases (NOXs), the main source of cellular ROS, I could confirm that not only the phosphorylation of EGFR but also the expression of fibrotic protein was decreased. I also reconfirmed the results using the newly tested materials in our lab, which interrupted EGFR phosphorylation.

Key words: Pulmonary fibrosis, ROS, EGFR

Ⅱ. INTRODUCTION

The family of interstitial lung diseases is characterized by cellular proliferation, interstitial inflammation, fibrosis, or a combination of such findings within the alveolar wall that is not due to infection or cancer. Idiopathic pulmonary fibrosis (IPF) is the predominant phenotype in most interstitial lung diseases [1]. It is characterized by recurrent injury of the alveolar epithelium with an unknown cause [2]. This can be considered to be a non-physiological scarring process where an excessive extracellular matrix (ECM) deposition leads to irreversible tissue damage and failure or disturbance of proper organ function [3]. IPF eventually results in progressive loss of lung function. Thereby, it is associated with high mortality and generally refractory to currently available pharmacological therapies.

However, it is becoming increasingly apparent that 'injured' and 'activated' alveolar epithelial cells both secrete and respond to growth factors themselves, particularly in IPF, thereby contributing to the outcome of the profibrogenic processes [4]. Among those growth factors, transforming growth factor (TGF)-β is considered a key molecule. Increased evidence indicates that production of reactive oxygen species (ROS) and oxidative stress are interlinked with TGF-β1 production and activation and thus are key to the fibrotic process [3].

One of the other growth factors, epidermal growth factor (EGF), is also overexpressed and associated with fibrotic progression in lung fibroblasts from IPF patients [5]. Meanwhile, TGF-β is known to induce the transactivation of the EGF receptor (EGFR) signaling pathway at least through the upregulation of EGFR [6]. Apart from the numerical

increment of EGFR, modification of EGFR by increased ROS stimulates its kinase activity, inducing EGFR signaling pathway [7].

Therefore, this research primarily focuses on TGF-β signaling pathway-induced ROS affecting EGFR activation and whether this effect has a definite influence on IPF.

Ⅲ. MATERIALS AND METHODS

Materials

TGF-β1 and EGF were purchased from Invitrogen (Waltham, Massachusetts, USA). These were used without further purification and dissolved in phosphate-buffered saline. 4',6,7-trimethoxyisoflavone (TMF) and catechol were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). These were used without further purification, and purities were determined using high-performance liquid chromatography. The TMF and catechol powder was dissolved in DMSO.

Cell culture

A549 cell line was stabilized and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) that was supplemented with 10% fetal bovine serum (FBS, Omega) and 1% penicillin/streptomycin (PAA). Cells were incubated in a humidified atmosphere at 37˚C in 5% CO_2.

Immunoblotting

A549 cells were seeded (3.5 ~ 4.5 * 10^5 each) on 60mm cell culture dishes and incubated for 30 minutes ~ 48 hours. The same amount of protein in each sample (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected using western blotting. ImageJ software was used to evaluate the intensity of the protein bands. The following primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA): anti-COL4A6

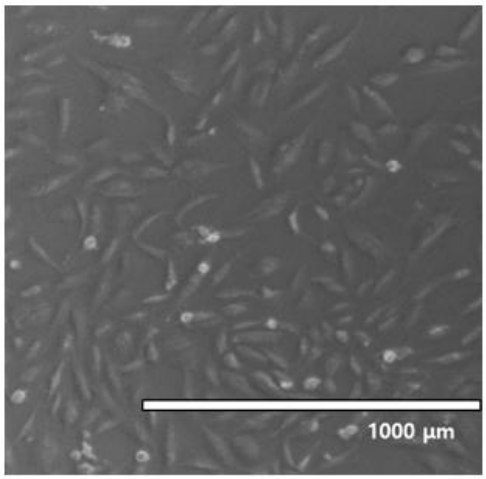
(SC-398655), anti-Fibronectin (SC-8422), anti-NOX1 (SC-25545), anti-NOX2 (SC-130543), anti-NOX4 (sc-1518092), anti-pERK (sc-7383) and anti-GAPDH (sc-25778) antibodies. And the following primary antibodies were purchased from Cell Signaling TECHNOLOGY (MA, USA): pEGFR (3777S) and pSMAD2 (3108S). The anti-a-SMA antibody was purchased from SIGMA-ALDRICH (A5228). Secondary antibodies (rabbit and mouse) were procured from Koma Biotech (Seoul, Republic of Korea).

Ⅳ. RESULTS

1. TGF-β1 and EGF affect the morphology of A549 cells, making those resemble myofibroblasts.

It is well known that TGF-β1 causes epithelial cells such as A549 to have morphology of activated myofibroblasts by allowing EMT to progress. In this experiment, I investigated whether EGF also has the similar effect on A549 as TGF-β1. Treating TGF-β1 (10 ng/ml) or EGF (2 ng/ml) upon A549 cells for 48 hours changed the morphology of them. The cells became stretched and lengthened like activated myofibroblasts as can be seen in the circles (Figure 1A, B, C), compared to the control group which was treated DMEM only.

(A)



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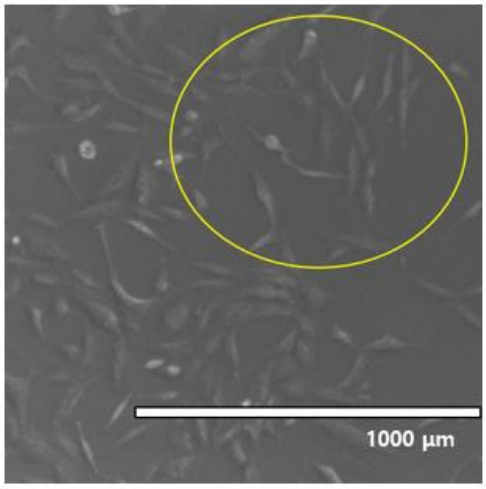

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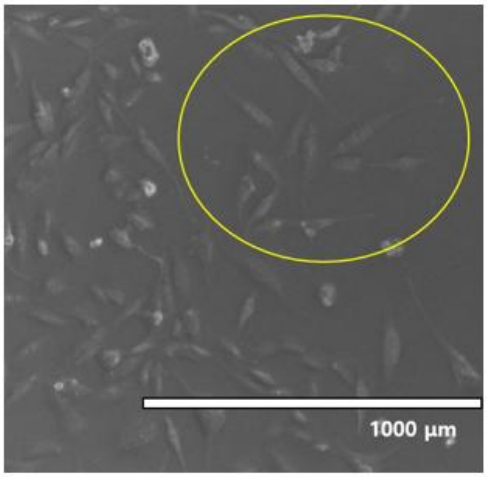


Figure 1. TGF-β1 and EGF promoted the change of the morphology of A549 cells.

Treating TGF-β1 (10 ng/ml) or EGF (2 ng/ml) upon A549 cells for 48 hours changed the morphology of them. **(A)** DMEM(2% FBS)-treated A549 cells. **(B)** TGF-β1 (10 ng/ml) included DMEM(2% FBS)-treated A549 cells. **(C)** EGF (2 ng/ml) included DMEM(2% FBS)-treated A549 cells. In the circles, cells which resemble activated myofibroblasts can be seen.

2. Not only TGF-β1 but also EGF can provoke fibrosis when treated upon A549 cells.

By confirming that EGF changes the morphology of A549 like TGF-β1 does, I saw the possibility that EGF treatment can induce fibrosis. I checked this through over-time western blot, which can show the changing trend of each factor's expression. Western blot results of over-time after treating TGF-β1 (10 ng/ml) upon A549 cells are shown here (Figure 2A, B). Given that increased expression of pSMAD2 and pERK, these results suggest that integration of Smad and non-Smad signaling made expression of fibrosis-specific markers such as fibronectin, collagen type 4 α 6 (COL4A6) and alpha smooth muscle actin (a-SMA) increase. In addition, western blot results of over-time after treating EGF (2 ng/ml) upon A549 cells are shown here (Figure 2C, D). I could confirm the effect of EGF by the increased expression of pEGFR. Increased expression of pERK, COL4A6 and a-SMA suggest that EGF also affected the fibrotic progression. Meanwhile, through the elevated expression of NADPH Oxidases (NOX1, 2, 4), EGF seemed to promote the generation of ROS and thereby proceeding fibrosis.

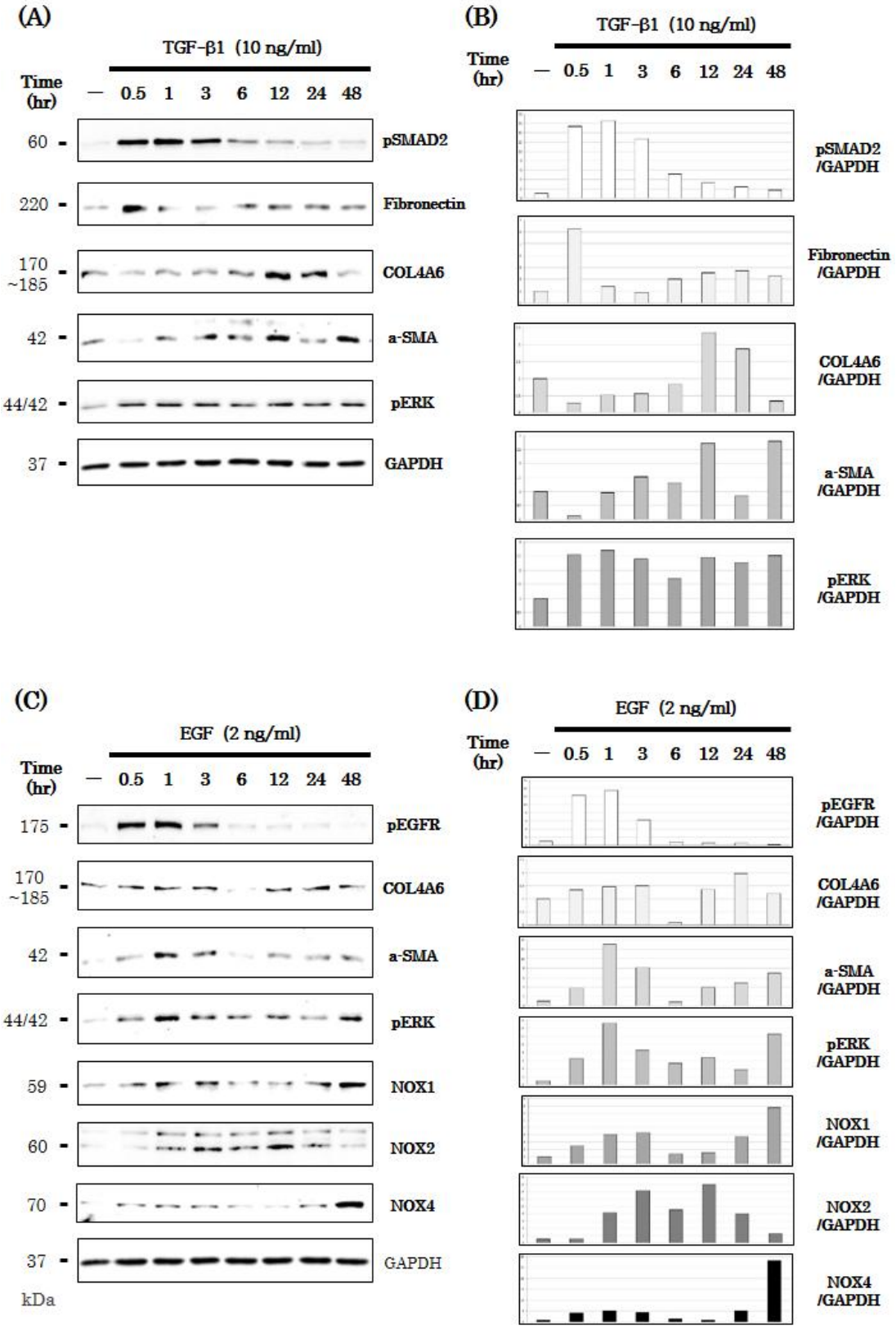


Figure 2. Both TGF-β1 and EGF provoked fibrosis when treated upon A549 cells.

Western blot results, showing over-time from 30 minutes to 48 hours after treating TGF-β1 (10 ng/ml) **(A)** or EGF (2 ng/ml) **(C)** upon A549 cells. Both TGF-β1 and EGF seem to promote fibrosis and in the case of EGF treatment, the expression of NOXs also increased. **(B)** and **(D)** show the relatively quantified graphs of (A) and (C) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

3. The progression of TGF-β1 signaling pathway upregulates the phosphorylation of EGFR, mediated by ROS.

In the previous experiments, it was confirmed that TGF-β1 signaling pathway and EGF signaling pathway independently induce fibrosis. Then, I checked the interaction between the two paths. It was confirmed through western blot that how the TGF-β1 signaling pathway induced by treating TGF-β1 affects the progress of the EGF signaling pathway. Western blot results of over-time after treating TGF-β1 (10 ng/ml) upon A549 cells are shown here (Figure 3A, B). TGF-β1 signaling pathway appeared to be provoking the EGF signaling pathway by upregulating the phosphorylation of EGFR. In this process, it is assumed that ROS generated from the progression of TGF-β1 signaling pathway is involved, given the increase in the expression of NOXs in Figure 3A along with the results from other earlier studies.

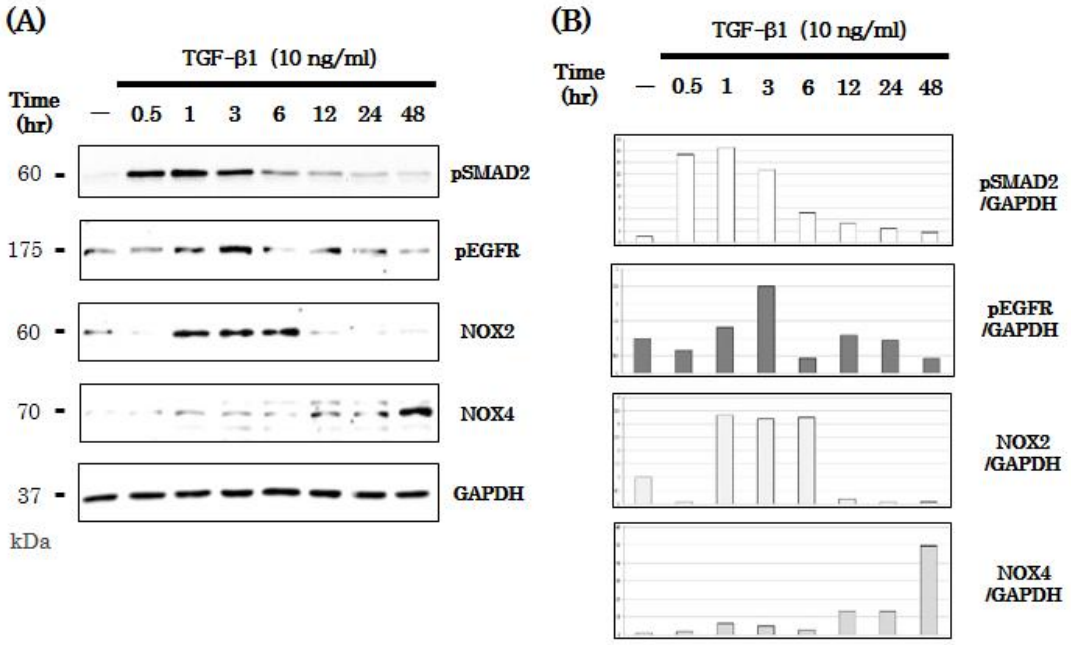


Figure 3. The phosphorylation of EGFR showed a subsequent increase to the progression of TGF-β1 signaling pathway.

(A) Western blot results of over-time after treating TGF-β1 (10 ng/ml) upon A549 cells. The expression of NOX2 and NOX4 increased over time, which may have led to an increase in ROS, resulting in the phosphorylation of EGFR. **(B)** shows the relatively quantified graphs of (A) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

4. Induced ROS directly by H_2O_2 affects phosphorylation of EGFR, resulting in fibrotic progression.

Increased evidence indicates that production of ROS and oxidative stress are interlinked with production and activation of various growth factors and cytokines, which are crucial for fibrosis development and persistence. In fact, in the previous experiments, it was confirmed that the expression of NOXs was increased by TGF-β1 or EGF. Accordingly, the increased ROS will be involved in many stages of fibrosis progression, including sulfenylation of EGFR, leading to fibrosis. To confirm this assumption, ROS was directly reacted with cells through H_2O_2 treatment. Western blot results of treating H_2O_2 (50 μM) upon A549 cells for 3 hours (Figure 4A, B) and 24 hours (Figure 4C, D) are shown here. ROS induced by H_2O_2 significantly increased the phosphorylation of EGFR and seemed to affect some fibrotic markers such as COL4A6 and pERK(for pERK, especially in 3 hours). Such effects of ROS were reaffirmed through inhibition by ROS scavenger (N-Acetyl Cysteine (NAC), 10 mM) and NOX inhibitors (Diphenyleneiodonium chloride (DPI) 10 μM, Apocynin 10 μM, GKT137831 5 μM). The ROS scavenger and NOX inhibitors were pre-treated 1 hour before H_2O_2 treating.

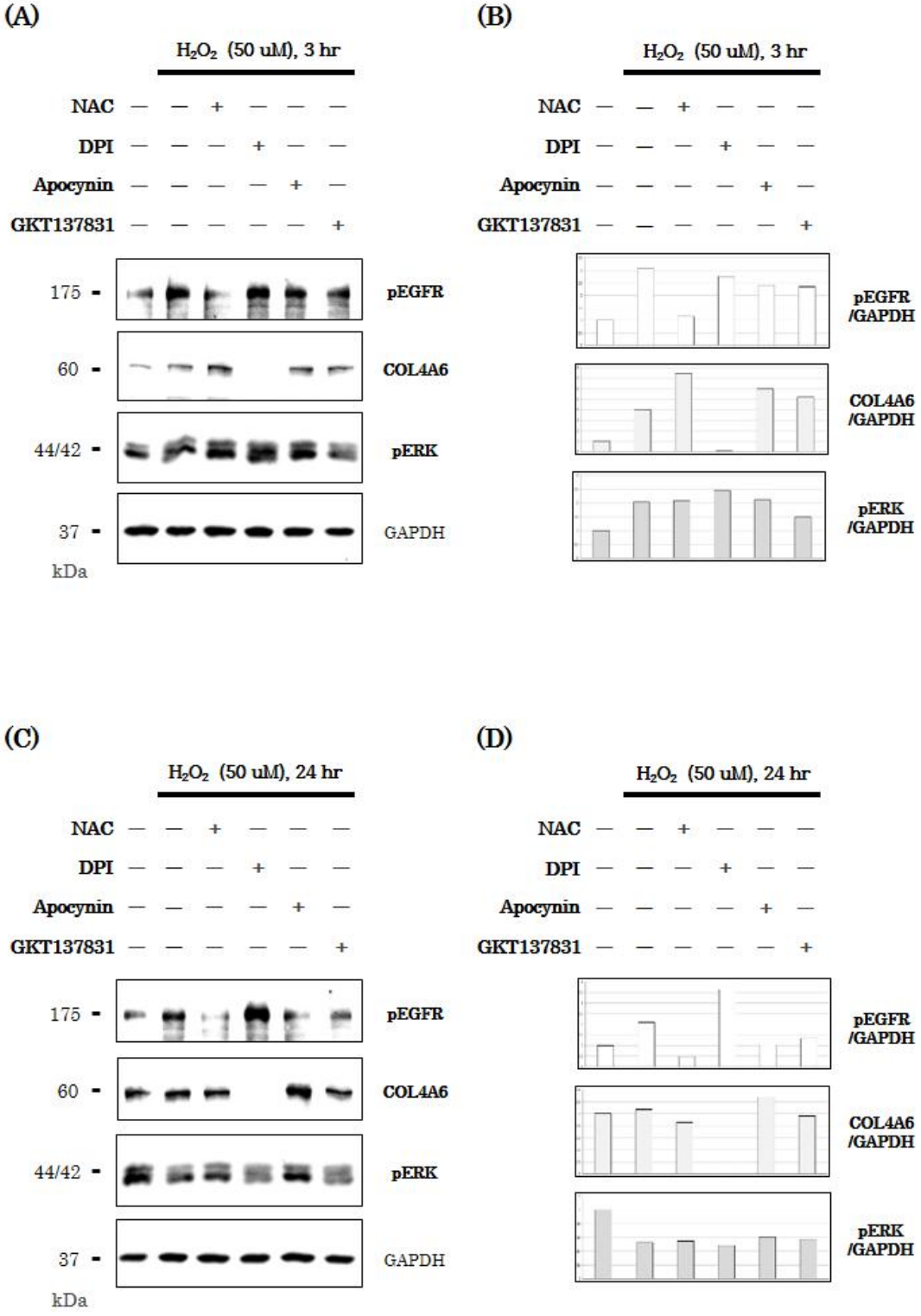
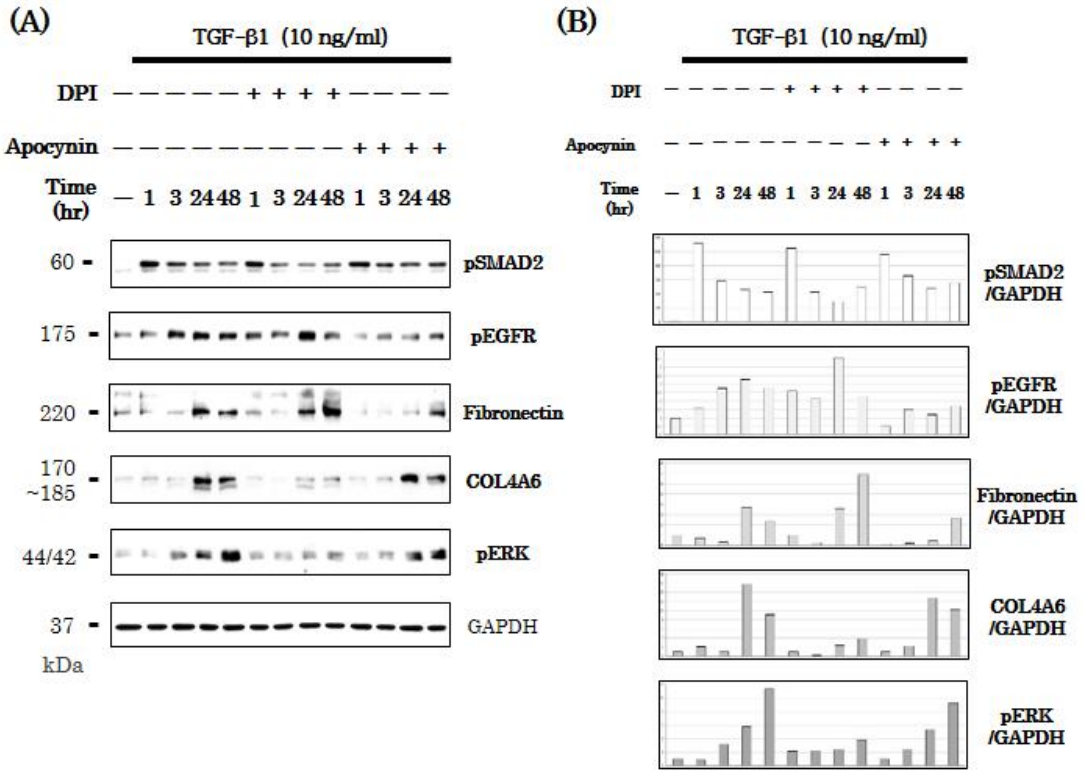


Figure 4. H_2O_2 increased phosphorylation of EGFR, again reduced by ROS scavenger and NOX inhibitors.

Western blot results of treating H_2O_2 (50 μM) upon A549 cells for 3 hours **(A)** and 24 hours **(C)**, after 1 hour pre-treatment of ROS scavenger (NAC) and NOX inhibitors (Apocynin, GKT137831), are shown here. The upregulating effects of ROS on EGFR and fibrotic proteins were confirmed in their increase over time and reduction by NAC, Apocynin and GKT137831. **(B)** and **(D)** show the relatively quantified graphs of (A) and (C) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

5. **NOX inhibitors attenuate both EGFR phosphorylation and fibrosis induced by TGF-β1 signaling pathway.**

As seen in the previous experiment, ROS is directly involved in the progression of fibrosis, which is known to be deeply involved in the progression of TGF-β1 signaling pathway, but this has been reaffirmed through the use of NOX inhibitors in terms of EGFR phosphorylation and fibrotic markers' expression. Western blot results of treating TGF-β1 (10 ng/ml) over-time upon A549 cells after 1 hour acclimation of NOX inhibitors (DPI (10 μM), Apocynin (10 μM)) (Figure 5A, B) are shown here. Depending on the type of NOX inhibitors, there are differences in the type of their inhibiting factors; in the case of DPI, mainly the expression of COL4A6 and pERK, and apocycin, pEGFR and fibronectin were affected. The significant inhibition of COL4A6 by DPI (10 μM) was re-proved in Figure 5C. The difference in mechanisms of action according to the type of NOX inhibitors needs to be studied further, but overall, it can be seen that the general fibrotic progression was alleviated by reducing ROS generation.



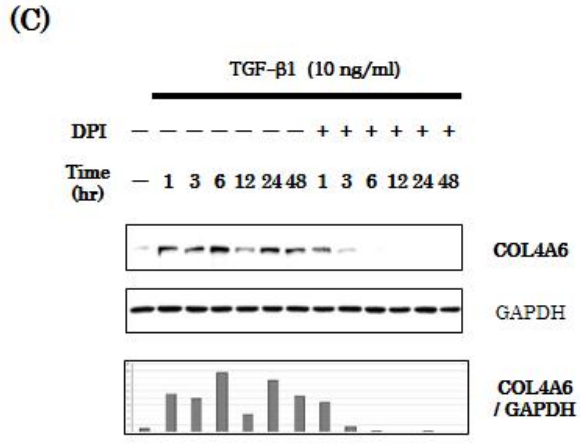


Figure 5. Under treatment of TGF-β1, NOX inhibitors attenuate both EGFR phosphorylation and fibrosis.

(A) Western blot results of treating TGF-β1 (10 ng/ml) over-time upon A549 cells after 1 hour acclimation of NOX inhibitors (DPI, Apocynin). Depending on the type of NOX inhibitors, there are differences in the type of their inhibiting factors. However, overall, it can be seen that the general fibrotic progression was alleviated by reducing ROS generation. **(C)** In the case of DPI, the significant inhibition of COL4A6 was re-proved. **(B)** and the graph of (C) show the relatively quantified graphs of (A) and (C) western blot results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

6. ROS scavenger and NOX inhibitors can attenuate EGFR phosphorylation which is stimulated by EGF treatment.

Whether ROS is directly involved in EGF signaling pathway as well as TGF-β1 signaling pathway, especially how it affects the phosophylation of EGFR, was confirmed through the use of ROS scavenger and NOX inhibitors. Western blot results of treating EGF (2 ng/ml) for 1 or 3 hours upon A549 cells, after 1 hour acclimation of ROS scavenger (NAC, 10 mM) or NOX inhibitors (DPI 10 μM, Apocynin 10 μM, GKT137831 5 μM), are shown here (Figure 6). Direct phosphorylation of EGFR by EGF is alleviated by the ROS scavenger and NOX inhibitors. In particular, the decrease was noticeable in groups treated with EGF for 3 hours. Considering the previous results of this study, those decrease in pEGFR will also contribute to inhibition of fibrosis.

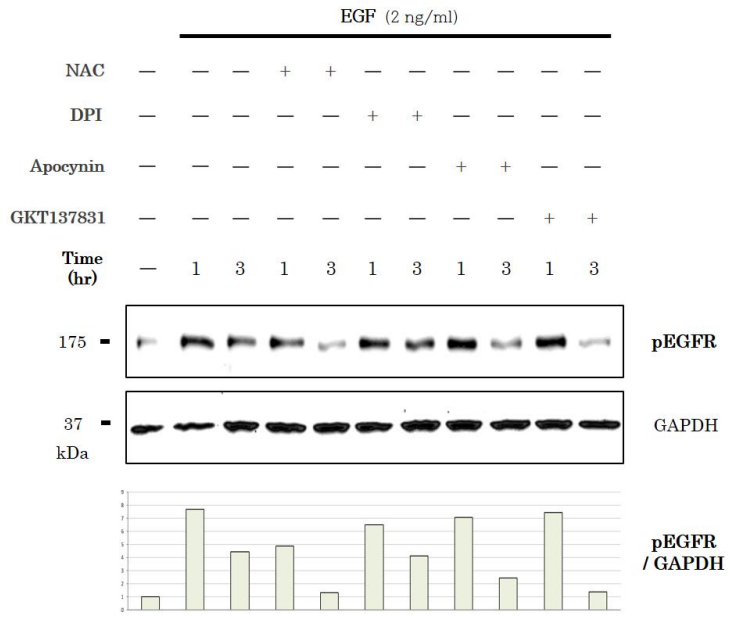


Figure 6. Under treatment of EGF, ROS scavenger and NOX inhibitors can attenuate EGFR phosphorylation.

Western blot results of treating EGF (2 ng/ml) for 1 or 3 hours upon A549 cells, after 1 hour acclimation of ROS scavenger (NAC) or NOX inhibitors (DPI, Apocynin, GKT137831). There were significant decreases in phosphorylation of EGFR in 3 hours EGF-treated groups. These effects will may have led to attenuation of fibrosis. The graph shows the relatively quantified graphs of western blot results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

7. TMF and catechol, which are believed to scavenge ROS, attenuate fibrosis.

4',6,7-trimethoxyisoflavone (TMF), which is a member of flavonoids, and catechol, which is a functional moiety responsible for the antioxidant effects of phenolic compounds, are believed to have inhibitory effect against ROS. Therefore, under TGF-β1 or EGF treatment conditions, TMF and catechol were used instead of the ROS scavenger or NOX inhibitors used earlier, and whether those show similar antifibrotic effects were observed.

1) Western blot results of treating TGF-β1 (10 ng/ml) for 24 or 48 hours upon A549 cells, together with TMF (2 μM) or catechol (2 μM), are shown here (Figure 7A, B). As shown by ROS scavenger or NOX inhibitors of the previous results, TMF and catechol reduced EGFR phosphorylation and the expression of fibrotic markers. They are assumed to act as ROS scavengers. Whether the single administration of TMF and catechol is better or co-administration is better varies slightly depending on the observing factor, but the antifibrotic effect was generally observed well in the co-administration.

2)
Western blot results of treating EGF (2 ng/ml) for 30 minutes or 1 hour upon A549 cells, together with TMF (2 μM) or catechol (2 μM), are shown here (Figure 7C, D). As with TGF-β1, TMF and catechol also reduced EGFR phosphorylation and the expression of fibrotic markers such as

pERK, when EGF was treated. In addition, they also reduced the expression of NOX1 and NOX2, contributing to the reduction of ROS generation itself.

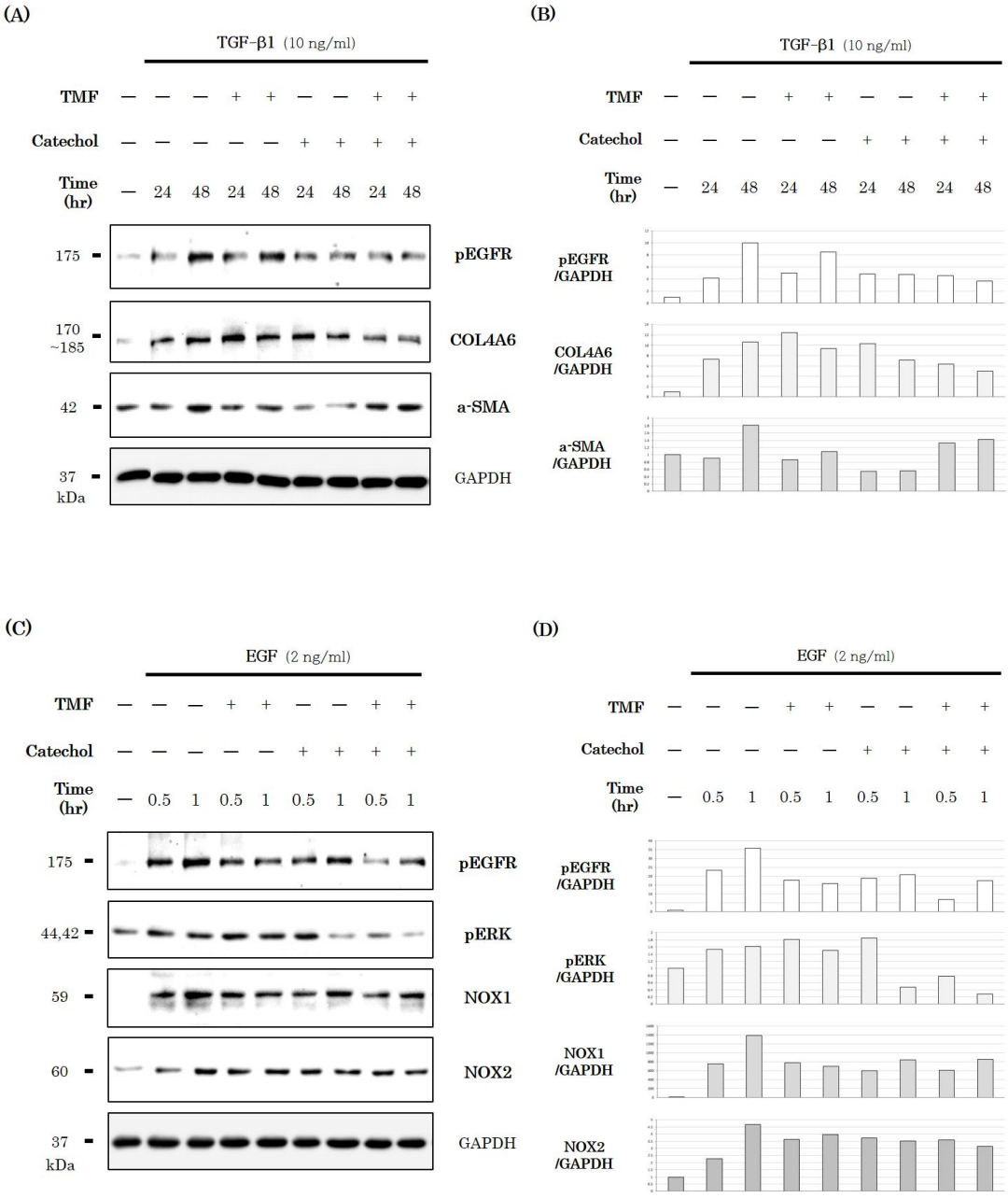


Figure 7. TMF and catechol can attenuate both phosphorylation of EGFR and fibrosis.

Western blot results of treating TGF-β1 (10 ng/ml) for 24 or 48 hours **(A)**, or EGF (2 ng/ml) for 30 minutes or 1 hour **(C)**, upon A549 cells, together with TMF (2 μM) or catechol (2 μM). As shown by ROS scavenger or NOX inhibitors of the previous results, TMF and catechol reduced EGFR phosphorylation and the expression of fibrotic markers. Those were also effective in reducing the expression of NOXs, contributing to the reduction of ROS generation itself. **(B)** and **(D)** show the relatively quantified graphs of (A) and (C) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

Ⅳ. DISCUSSION

Traditionally, many studies have conducted *in vitro* fibrosis induction by TGF-β1. And for fibrosis induced by TGF-β1, the order of the pathway and the range of influence of the process were studied in detail. Fibrosis by TGF-β1 forms a kind of fibrotic niche rather than a linear process because during the process of fibrosis by TGF-β1, a lot of other types of growth factors and cytokines are produced and secreted by the cells. Thus I thought more research on fibrosis progression subsidized by growth factors other than TGF-β1 was needed.

According to that need, I further explored the fibrosis induction by EGF. In this study, it seems that EGF signaling pathway activated by EGF treatment may induce fibrosis by itself, and EGF signaling pathway is also activated by TGF-β1 treatment, resulting in acceleration of fibrosis. In order to more clearly confirm the effect of EGF signaling pathway contributing to fibrosis, I can check the degree to which fibrosis is induced, when TGF-β1 is treated and an EGFR blocker is used, compared with the non-EGFR blocker group.

Meanwhile, in the interaction of two paths that contribute to fibrosis; TGF-β1 signaling pathway and EGF signaling pathway, it was noted that ROS can be the main mediator between those. Increased evidence indicates that production of ROS and oxidative stress are interlinked with production and activation of various growth factors and cytokines, which are crucial for fibrosis development and persistence. TGF-β1 was shown to promote ROS formation mainly via induction of NOX4 expression and hence activity in various types of cells. The ROS produced in response to TGF-β1 is able to activate various signaling

pathways, thereby influencing gene transcription networks downstream from TGF-β1. ROS also affects the EGF signaling pathway, especially in the activation of EGFR. The modification of EGFR by increased ROS at a critical cysteine (Cys797) in its catalytic site stimulates its kinase activity, thereby demonstrating that sulfenylation can regulate receptor tyrosine kinase (RTK) function [7].

In order to confirm the influence of ROS as above in the *in vitro* A549 experiments, first, the generation of ROS when TGF-β1 or EGF is treated can be confirmed through DCFDA assay. In addition, NOX expression can be identified through western blot. And in order to confirm the contribution of EGF signaling pathway to the process of fibrosis directly induced by ROS, the EGFR blocker-used group can be compared with the non-EGFR blocker group. It is also necessary to explore the process of fibrosis which is a result of the effect of ROS on EGFR sulfenylation, in more detail. To this end, it should be checked whether the sulfenylation of EGFR is actually increased when EGF, TGF-β 1, or ROS is treated, and whether that increased sulfenylation actually become decreased when ROS scavenger is additionally treated. In addition, it is also necessary to observe the change in the degree of fibrosis according to the increase or decrease in sulfenylation.

About TMF and catechol, our lab is reasoning specific mechanism of action (MOA), but it needs to be explored clearly through more diverse experiments. It should be studied whether they actually inhibit fibrosis, what would be their ideal combination ratio for inhibition, what would be their MOA, and what would be the difference from other ROS scavengers.

Ⅴ. CONCLUSION

The process from TGFβ-TGFβR bingding to EGFR phosphorylation and the interplay with ROS of this process were investigated in this study (Figure 8). Herein the results suggest that not only the EGF signaling itself, but also the subsequent activation of EGFR by TGF-β signaling pathway can accelerate fibrosis. Further it has been highlighted that ROS plays a major role in such EGFR activation, which expects antioxidants to work as fibrosis treatments. From this point of view, TMF and catechol are being studied as medicine candidates, and it is urgent to reveal their MOAs.

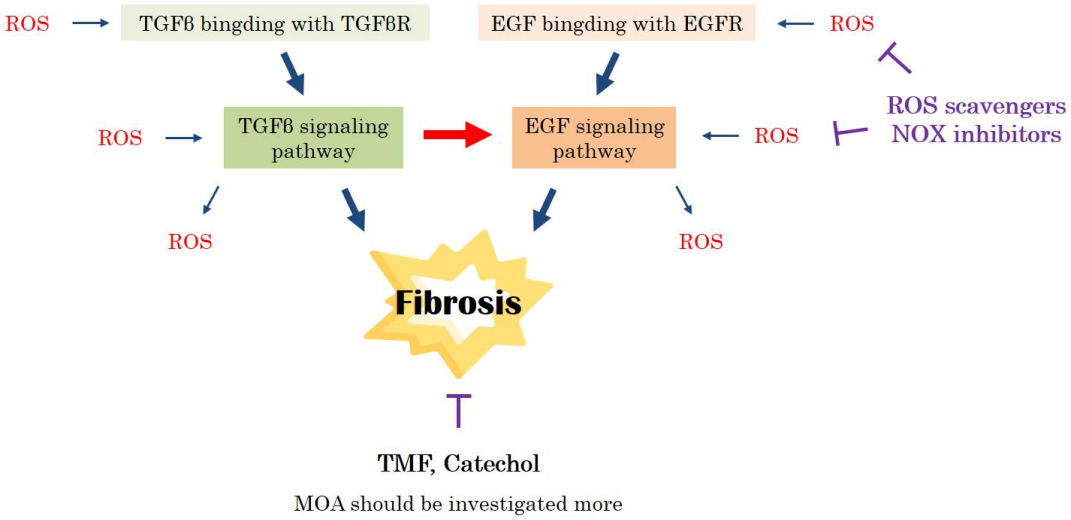


Figure 8. Overview of this study.

Through ROS, TGF-β signaling pathway affects EGF signaling pathway, especially the phosphorylation of EGFR, which results in acceleration of fibrosis.

PART 2.

TMF/catechol co-administration can be a therapeutic candidate for bleomycin-induced pulmonary fibrosis, by inhibiting EMT

Ⅰ. ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a fatal lung disorder with a median survival of only 3 years and remains a clinical dilemma. IPF is characterized by abnormal extracellular matrix (ECM) deposition by activated myofibroblasts. While the origin of myofibroblasts has been discussed for many years, the epithelial-mesenchymal transition (EMT) is being noticed as one of the mechanisms of myofibroblast activation.

To evaluate the effects of 4′,6,7-trimethoxyisoflavone (TMF) and catechol on EMT and fibrosis, we used an *in vitro* transforming growth factor (TGF)-β1-induced model and an *in vivo* bleomycin (BLM)-induced model. Results showed that co-administration of TMF/catechol ameliorated the established pulmonary fibrosis by suppressing EMT and ECM accumulation through hindering both Smad and non-Smad TGF-β signaling cascades. Further, a significant increase in the level of inflammatory cytokines (IL-1β, IL-13, and TNF-α) of BLM-treated mice' lungs was put down with TMF/catechol co-treatment, compared to control group.

Our findings suggest that co-intervention using TMF/catechol might be a potential therapeutic candidate for the treatment of pulmonary fibrosis.

Key words: Pulmonary fibrosis, EMT, TMF, Cathechol, TGF-β1. BLM

Ⅱ. INTRODUCTION

Fibrosis is an outcome of the tissue repair response that becomes dysregulated following many types of tissue injury, most notably during chronic inflammatory disorders. When the injury with unknown cause against the alveolar epithelium is repetitive or severe, it becomes idiopathic pulmonary fibrosis (IPF). Activated and proliferating fibroblasts continue to secrete extracellular matrix (ECM) components, which can lead to disruption of lung architecture, dysfunction and ultimately failure [8].

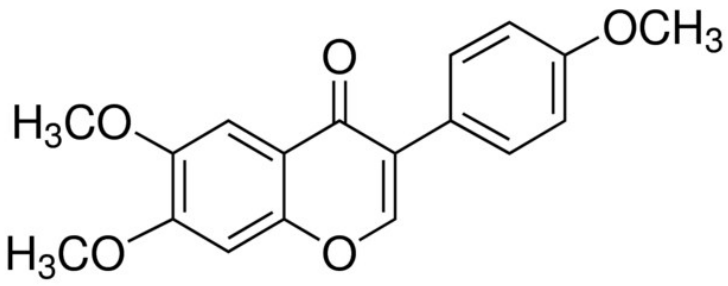
In addition to activation of fibroblasts and immune cells, fibrosis-inducing events also cause release of profibrotic metabolites (e.g., reactive oxygen species (ROS)), and secretion of chemokines and growth factors. Increased evidence indicates that production of ROS and oxidative stress are interlinked with production and activation of various growth factors and cytokines; thereby feed-back as well as feed-forward cycles appear to exist [3].

Among the substances known to have antioxidant properties, flavonoids are widely found in the plant kingdom occurring commonly in the leaves, floral organs and pollen grains. Several lines of evidence corroborate the hypothesis that the flavonoids have antioxidant functions in higher plants that are challenged with a range of environmental stresses. As potent antioxidants, they can modulate a variety of disease conditions such as thrombosis, carcinogenesis and hepatotoxicity [9]. In phytochemical studies, isoflavonoids and phenolic acids were identified in the roots and stem bark. 4',6,7-trimethoxyisoflavone (TMF) (Figure 1A) is a chemically transformed product of an amphiisoflavone isolated from

the roots of the medicinal plant, *Amphimas pterocarpoides* [10]. Meanwhile, it is known that several phenolic compounds scavenge ROS and the functional moiety responsible for the antioxidant effects of those is the catechol group [11] (Figure 1B).

Herein I studied whether TMF and catechol alone or their co-treatment has any effect in decreasing expression level of mesenchymal cell specific markers and fibrotic proteins, using *in vitro* transforming growth factor (TGF)-β1 or bleomycin (BLM)-induced experiments and *in vivo* BLM-induced pulmonary fibrosis model. The change of inflammatory cytokines expression was also investigated *in vivo*.

(A)



(B)

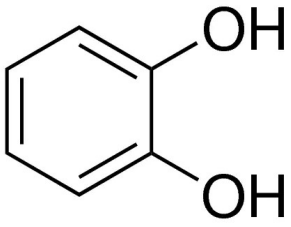


Figure 1. The chemical structures of TMF and cathechol.

(A) 4',6,7-trimethoxyisoflavone (TMF)

(B) catechol

Ⅲ. MATERIALS AND METHODS

Materials

4',6,7-trimethoxyisoflavone (TMF) and catechol were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). These were used without further purification, and purities were determined using high-performance liquid chromatography. The TMF and catechol powder was dissolved in DMSO. TGF-β1 and EGF were purchased from Invitrogen (Waltham, Massachusetts, USA). These were used without further purification and dissolved in phosphate-buffered saline.

Cell culture

A549 cell line was stabilized and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) that was supplemented with 10% fetal bovine serum (FBS, Omega) and 1% penicillin/streptomycin (PAA). Cells were incubated in a humidified atmosphere at 37˚C in 5% CO_2.

MTT assay

Cell viability/cytotoxicity was determined using a 3-(4, 5-dimethyl-2 -thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) assay). Cells were seeded into 96-well culture plates at a density of 3000 cells per well and then treated with TMF (2 μM) or catechol (2 μM). After different times of incubation, the assay was performed by adding 10 μL of MTT solution (5 mg/mL in PBS; Sigma-Aldrich) to each well, followed by incubation for 4 h. The crystals were dissolved with 200 μL of dimethyl sulfoxide and the

formazan crystal production was quantified using a spectrophotometer (562 nm).

Western Blotting and Antibodies

A549 cells were seeded (3.5 ~ 4.5 * 10^5 each) on 60mm cell culture dishes and incubated for 30 minutes ~ 48 hours. Cells were washed twice with PBS and harvested in RIPA buffer. Total protein amounts were measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). The same amount of protein in each sample (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected using western blotting. ImageJ software was used to evaluate the intensity of the protein bands. The primary antibodies used included those against Fibronectin, Actin, pERK, NOX1, NOX2, NOX4, GAPDH, pP38MAPK, TGF-β1 (Santa Cruz Biotechnology, Dallas, TX. USA), pSMAD2/3, E-cadherin, Vimentin, pAKT, pEGFR (Cell Signaling Technology, Beverly, MA, USA) and alpha smooth muscle actin (SIGMA-ALDRICH, MO, USA). Secondary antibodies (rabbit and mouse) were procured from Koma Biotech (Seoul, Republic of Korea).

Immunofluorescence

Cells grown on Lab Tek eight-well chamber slides at an initial cell seeding density of 2 * 10^5 cells/well were fixed with 4% buffered paraformaldehyde in PBS and washed three times with PBS. Fixed cells were then permeabilized with 0.3% triton X-100 and incubated in 5% bovine serum albumin for 1 h. Samples were incubated with the primary antibody overnight at 4 ℃ and subsequently incubated with a secondary antibody conjugated with Alexa Fluor 595, Alexa Fluor 488, or Texas red

for 1 h at room temperature. After several washes with PBS, the slides were incubated with DAPI for 5 min and then mounted in glycerol. Images were photographed and analyzed using an EVOS system (Advanced Microscopy Group, Bothell, WA, USA).

Transwell migration assay

A549 cells were serum-starved with DMEM containing 1% FBS overnight. Cells ($6*10^4$/well) were seeded on Matrigel-coated and uncoated Transwell inserts with a 8-μm pore size and then transferred to the lower chamber in a 24-well plate containing medium supplemented with 2% FBS (negative control) with/without 10 ng/mL TGF-β1 or 20 μg/mL Bleomycin (stimulant, positive control). The wells were incubated for 24 h in a 37℃ CO_2 incubator and the Transwell inserts were then removed from the 24-well plate. Remaining cells that had not migrated from the top of the insert were removed with wet cotton swabs and samples were fixed with 4% paraformaldehyde for 10 min and stained with crystal violet.

Sirius red staining

A549 cells were seeded into 96-well culture plates at a density of 5000 cells per well and then in the mid of the process, after 2 and 4 days after seeding, the cells were serum-starved with DMEM containing 2% FBS and treated with 20 μg/mL bleomycin or TMF (2 μM) and catechol (2 μM) containing 1% FBS. After 5 days of treating, the cells were fixed with 4% buffered paraformaldehyde in PBS. Then those were fixed with methanol o/n and staining solution was added 50 ul per well for 1 hr.

Determination of ROS generation

Intracellular ROS levels were determined using 20,70-dichlorofluorescein diacetate (DCFDA) assay. After respective treatment for the indicated period, the cells were incubated with DCFDA (10 μM) in complete medium for 30 min at 37℃. The emitted fluorescence was read in a microplate spectrophotometer plate reader (GENios, TECAN Group, Maennedorf, Switzerland) at Ex/Em 502/535 nm.

Histopathological analysis

Hematoxylin and eosin (H&E) and Masson's trichrome staining was performed on lung tissues. H&E-stained and trichrome stained lung images were acquired at different sites at three magnifcations using an Olympus BX51 microscope. Based on those images, ashcroft score was valued. Each successive field was individually assessed for severity of interstitial fibrosis and allotted a score between 0 and 8 using a predetermined scale of severity. After examining the whole section the mean score of all the fields was taken as the fibrosis score for the section.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from FLSs using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesised by using a reverse transcriptase kit (Promega, Seoul, Korea).

Real-time polymerase chain reaction

Total RNA extraction and cDNA synthesis were performed as previously described. Real-time PCR was performed using SYBR Green Master mix (KAPA BIOSYSTEMS, Cape Town, South Africa) according to the manufacturer's instructions.

ELISA

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated for 16 h at 4 ℃ with 2 μg/ml of rh LAP1, Latent TGF-β1 or TGF-β1 (R&D Systems) in 100 μl PBS. Other assay steps were at room temperature (RT), using 100 μl/well. Five washes using PBS with 0.1% Tween 20 were made between assay steps. After coating, wells were blocked for 1 h with incubation buffer (PBS with 0.05% Tween 20 and 0.1% bovine serum albumin). Hybridoma supernatants were diluted in incubation buffer and incubated 2 h. Next, goat-anti-mouse IgG alkaline phosphatase conjugate (Mabtech, Nacka Strand, Sweden) was added and incubated for 1 h, followed by development with para-nitrophenyl phosphate (Sigma) and absorbance measurement (405 nm) by an ELISA reader (Labsystems, Helsinki, Finland).

BALF analysis

Mice were anesthetized and lavage was performed using a 1 ml syringe with five consecutive cycles of filling and emptying with pre-warmed (37 ℃) phosphate-buffered saline (PBS) for cycle 1 and PBS containing 0.325% bovine serum albumin for cycles 2-5. Each cycle involved filling and emptying with 1 ml of lavage medium, which was then repeated. The supernatant from cycle 1 was obtained by 300 ×g centrifugation for 5

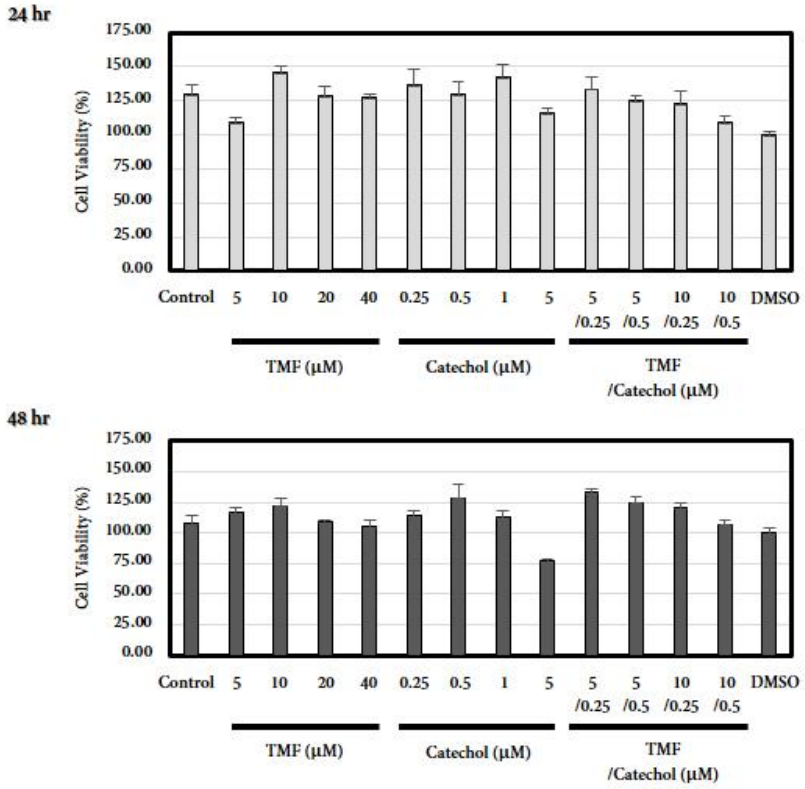
min at 4 ℃, then frozen in aliquots at −70 ℃ . Samples of bronchoalveolar lavage fluid (BALF) leukocytes (free lung cells, FLCs), contained in the cell pellet of cycle 1 combined with the complete cell suspension of cycles 2-5 were stored at 4 ℃ to characterize and enumerate cell subpopulations.

Ⅳ. RESULTS

1. TMF and catechol do not adversely affect the viability of several types of cell lines.

The MTT assays were done in order to check the effects of TMF and catechol against the viability of several types of cell lines. First, for the A549 cell line, TMF and catechol were treated at concentrations of 5 to 40 μM and 0.5 to 5 μM, respectively, and for the co-treatment, concentrations of 5/0.25, 5/0.5, 10/0.25, and 10/0.5 μM. The MTT assay results at 24 hours and 48 hours were observed for the same experimental groups. As a result, TMF and catechol did not significantly affect cell viability in all treatment groups except catechol 5 μM at 48 hours (Figure 2A). For other types of cells, such as myoblast (C2C12) and fibroblast (HDF, MRC-5), TMF and catechol were also examined by MTT assay to see if they affected their viability; the concentrations of TMF and catechol were 10 and 0.5 μM for single treatment and 10/0.5 μM for co-treatment. Here, TMF and catechol did not significantly affect at both 24 hours and 48 hours (Figure 2B).

(A)



(B) Control(Ctrl), TMF; 10 μM, Catechol(Cat); 0.5 μM, TMF+Cat; 10 μM + 0.5 μM

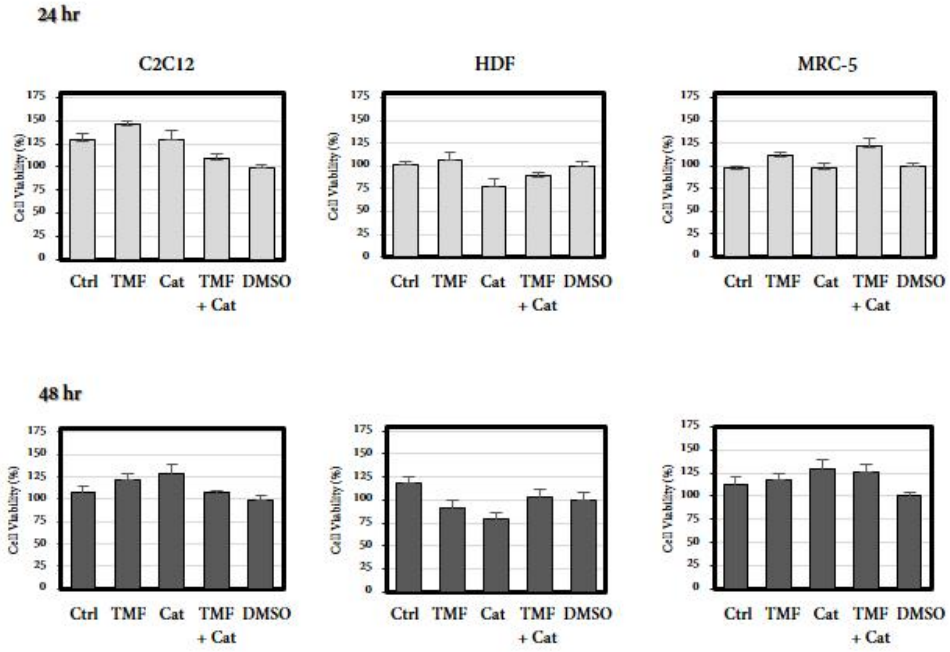


Figure 2. Various concentrations of TMF and catechol do not damage the cell viability.

(A) First, for the A549 cell line, TMF and catechol were treated at concentrations of 5 to 40 μM and 0.5 to 5 μM, respectively, and for the co-treatment, concentrations of 5/0.25, 5/0.5, 10/0.25, and 10/0.5 μM. The MTT assay results at 24 hours and 48 hours were observed and TMF and catechol did not significantly affect cell viability. **(B)** For other types of cells, such as myoblast (C2C12) and fibroblast (HDF, MRC-5), the concentrations of TMF and catechol were 10 and 0.5 μM for single treatment and 10/0.5 μM for co-treatment. Here, TMF and catechol did not significantly affect at both 24 hours and 48 hours.

2. TMF and catechol show inhibitory effect on TGF-β1-induced fibrosis by inhibiting EMT in A549 cells.

1)

In order to find out the efficacy of TMF and catechol on fibrosis, *in vitro* experiments using TGF-β1 were first conducted. Considering the expression time zone of the fibrosis markers (e.g. Fibronectin, COL4A6, a-SMA), two time zones: 24 hours and 48 hours were selected to treat TGF-β1. Western blot results of treating TGF-β1 (10 ng/ml) for 24 or 48 hours upon A549 cells, together with TMF (2 μM) or catechol (2 μM), are shown here (Figure 3A, B). TMF and catechol reduced EGFR phosphorylation and the expression of fibrotic markers. Whether the single administration of TMF and catechol is better or co-administration is better varies slightly depending on the observing factor, but the antifibrotic effect was generally observed well in the co-administration.

2)

By specifying 24 hours, changes in expression of various fibrosis specific markers and mesenchymal cell specific markers were observed again with western blot. From these results, it can be seen that TMF and catechol are effective in fibrosis mitigation and epithelial-mesenchymal transition (EMT) inhibition (Figure 3C, D). In addition, the mitogen-activated protein kinase (MAPK) signaling pathway specific markers were also observed, and TMF and catechol showed similar inhibitory effects (Figure 3E, F).

3)

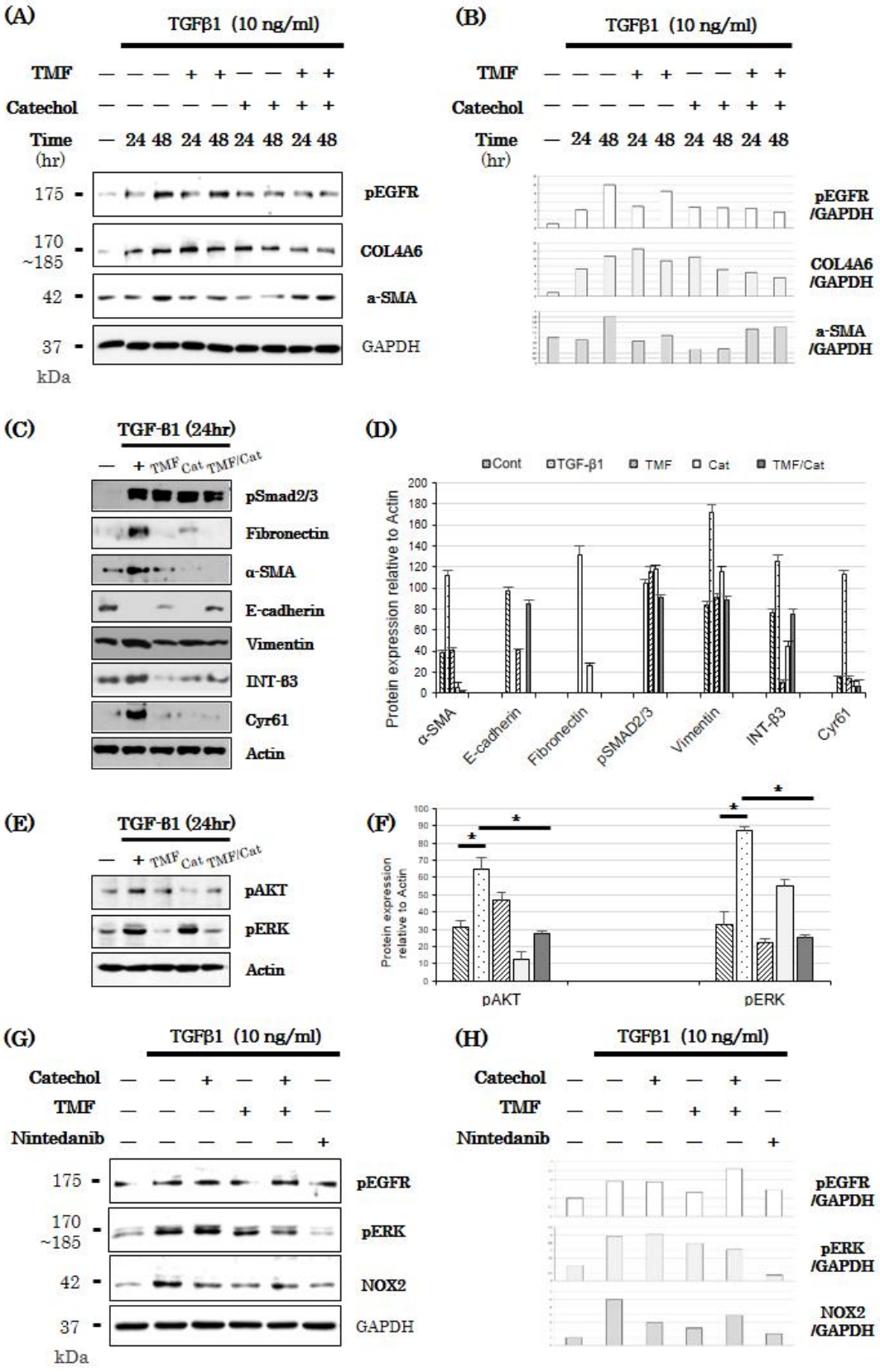
In addition, by specifying 48 hours, changes in expression of pEGFR, pERK and NOX2 were observed. TMF and catechol showed results in line with the previous results for pEGFR and pERK, and the expression of NOX2 was also reduced, thereby reducing the generation of ROS itself (Figure 3G, H). Their antifibrotic effects can be compared to nintedanib, one of only two FDA-approved drugs available for treating IPF.

4)

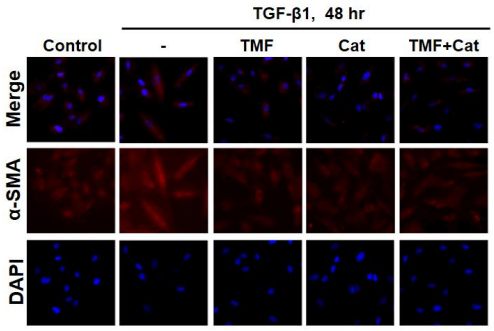
Their inhibitory effects on the fibrosis induced by TGF-β1 was also confirmed through immunofluorescence. As can be seen in Figure 3I, TMF and catechol reduced the expression of a-SMA which was increased by TGF-β1. It can be assumed that this effect was possible by inhibiting the EMT of A549 cells, which can be confirmed in Figure 3J. TMF and catechol were effective in restoring the expression of E-cadherin which was reduced by TGF-β1.

5)

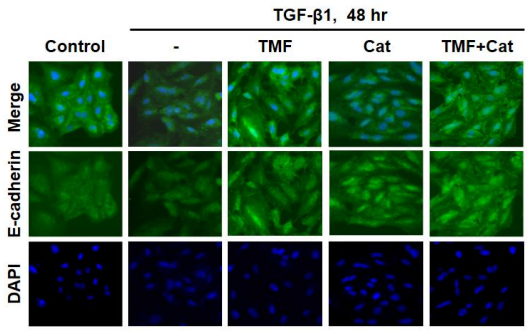
The EMT inhibition effect of TMF and catechol was re-proved through transwell migration assay, which can measure the mobility of A549 cells under treatment of TGF-β1, TMF or catechol. When TGF-β1 was treated, it could be seen that the migration of the cells increased in response to that, and this movement was inhibited by TMF and catechol (Figure 3K, L).



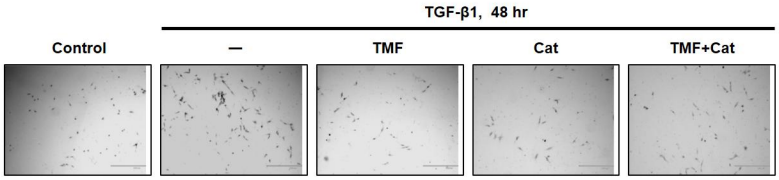
(I)



(J)



(K)



(L)

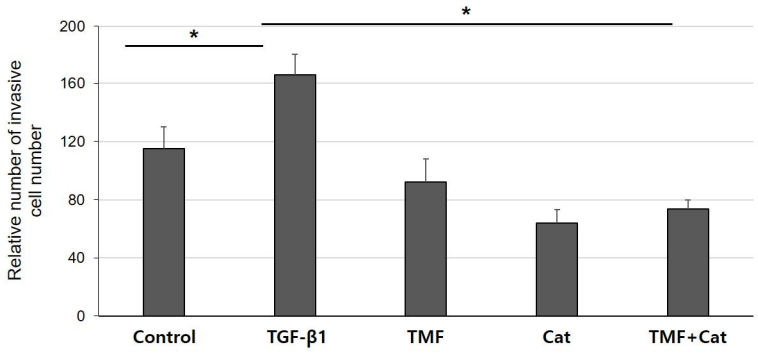


Figure 3. By inhibiting EMT, TMF and catechol showed attenuating effect on TGF-β1-induced fibrosis in A549 cells.

(A) Western blot results of treating TGF-β1 (10 ng/ml) for 24 or 48 hours upon A549 cells, together with TMF (2 μM) or catechol (2 μM). TMF and catechol reduced EGFR phosphorylation and the expression of fibrotic markers. **(B)** shows the relatively quantified graphs of (A) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression. **(C, D)** By specifying 24 hours, changes in expression of various fibrosis specific markers and mesenchymal cell specific markers were observed again with western blot. TMF and catechol are effective in inhibiting fibrosis and epithelial-mesenchymal transition (EMT). **(E, F)** The mitogen-activated protein kinase (MAPK) signaling pathway specific markers' expression were also inhibitied by TMF and catechol. **(G, H)** By specifying 48 hours, changes in expression of pEGFR, pERK and NOX2 were observed. TMF and catechol showed results in line with the previous results for pEGFR and pERK, and the expression of NOX2 was also reduced, thereby reducing the generation of ROS itself. **(I, J)** Their inhibitory effect on the fibrosis induced by TGF-β1 was also confirmed through immunofluoresce. TMF and catechol reduced the expression of a-SMA (I) and it can be assumed that this effect was possible by inhibiting the EMT (J). **(K, L)** The EMT inhibition effect of TMF and catechol was re-proved through transwell migration assay. When TGF-β1 was treated, it could be seen that the migration of the cells increased in response to that, and this movement was inhibited by TMF and catechol.

3. TMF and catechol show their effects also on EGF-induced fibrosis markers' and NOXs' expression in A549 cells.

In Part 1, it was confirmed that *in vitro* fibrosis can be induced by EGF treatment. For this EGF-induced fibrosis, TMF and catechol were also tested to see if they had efficacy. Western blot results of treating EGF (2 ng/ml) for 30 minutes or 1 hour upon A549 cells, together with TMF (2 μM) or catechol (2 μM), are shown here (Figure 4A, B). As with TGF-β1, TMF and catechol also reduced EGFR phosphorylation and the expression of fibrotic marker such as pERK, when EGF was treated. In addition, they also reduced the expression of NOX1 and NOX2, contributing to the reduction of ROS generation itself. With different time zones, the effects of TMF and catechol were also observed when EGF was treated for 24 or 48 hours (Figure 4C, D). Here, too, they reduced the expression of a-SMA, a fibrotic protein, and in this process, it is presumed that the EGFR phosphorylation they made decrease was involved.

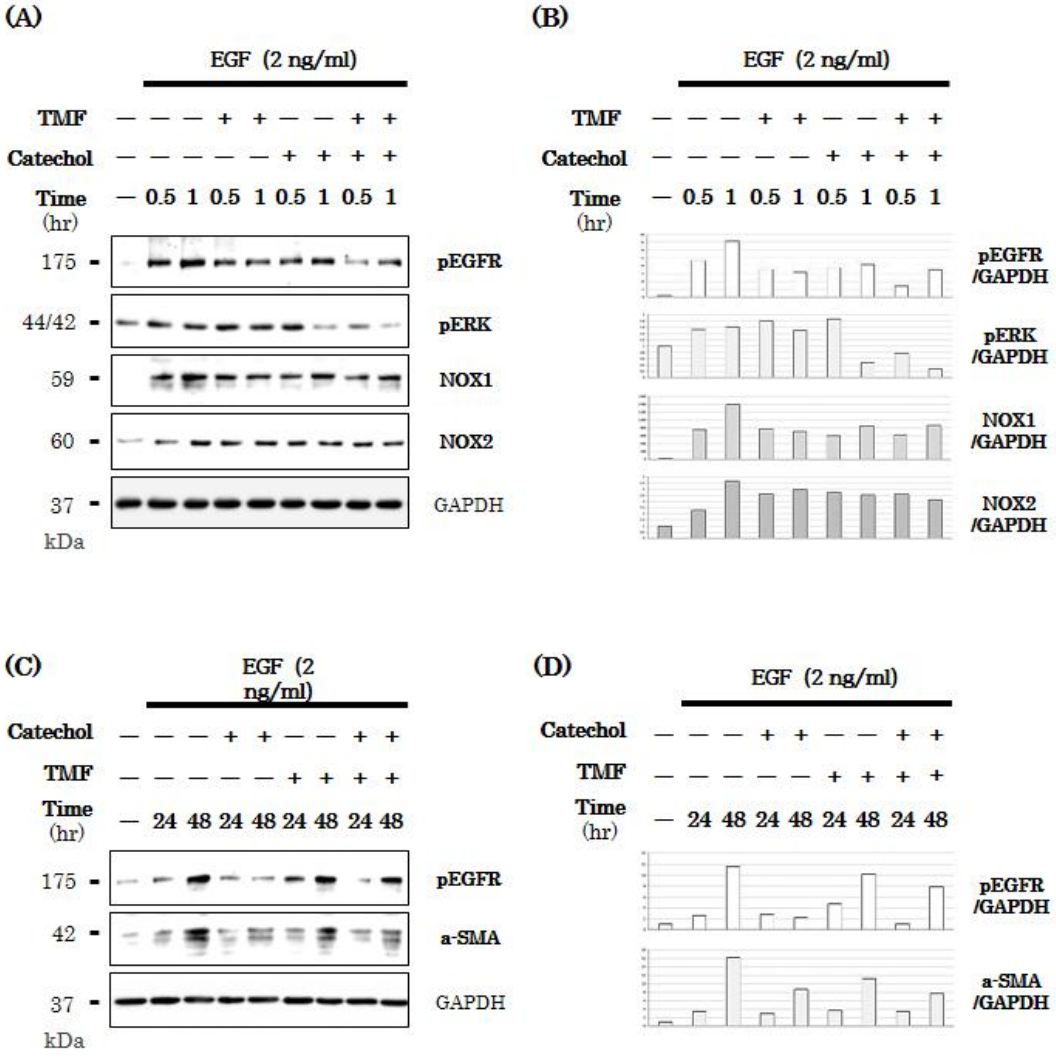
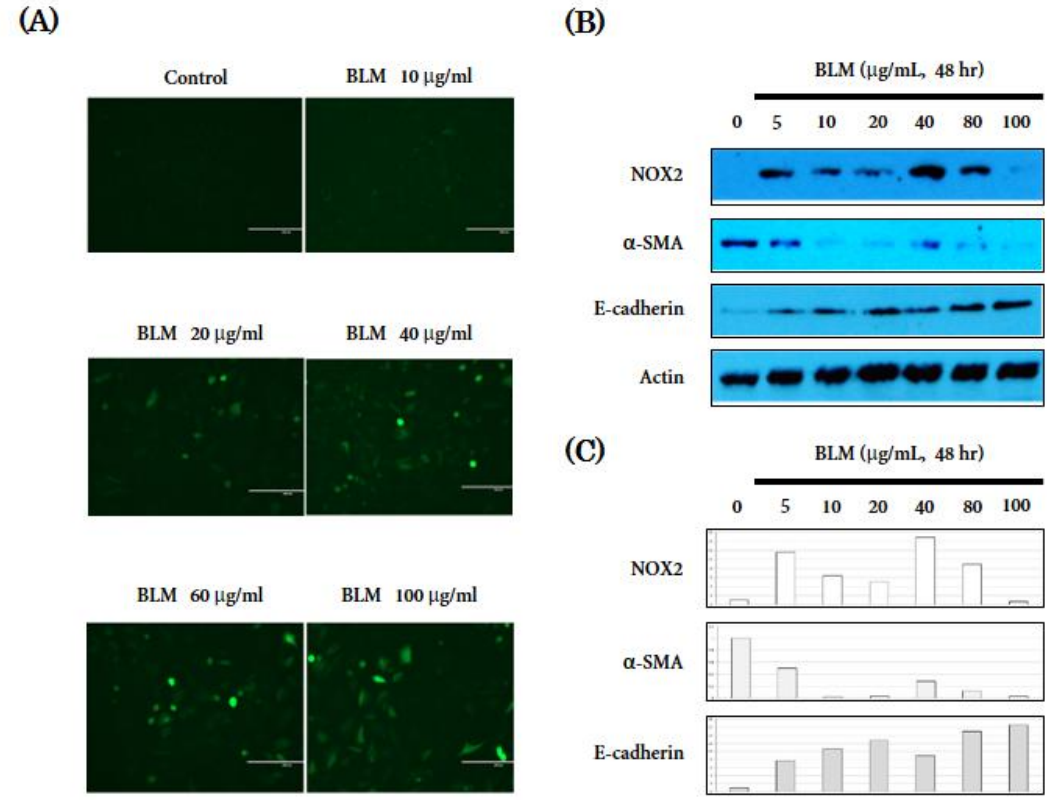


Figure 4. Under EGF-induced fibrosis, TMF and catechol showed their inhibitory effects against expression of fibrosis markers and NOXs in A549 cells.

(A) Western blot results of treating EGF (2 ng/ml) for 30 minutes or 1 hour upon A549 cells, together with TMF (2 μM) or catechol (2 μM). TMF and catechol reduced EGFR phosphorylation and the expression of fibrotic marker such as pERK. In addition, they also reduced the expression of NOX1 and NOX2. **(B)** shows the relatively quantified graphs of (A) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression. **(C)** The effects of TMF and catechol were also observed when EGF was treated for 24 or 48 hours. Here, too, they reduced the expression of a-SMA, and in this process, it is presumed that the EGFR phosphorylation they made decrease was involved. **(D)** shows the relatively quantified graphs of (C) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression.

4. BLM increases ROS with NOX2 expression, resulting in EMT and fibrosis in A549 cells.

It is well known that pulmonary fibrosis by BLM occurs in the *in vivo* model, and its induction method is also well established. However, the effects of BLM in *in vitro* experiments are not well known. Therefore, whether such BLM induces fibrosis *in vitro* and whether similar results are caused by BLM, just as ROS generation and expression of NOXs increase in *in vitro* fibrosis by TGF-β1, were investigated here. First, how the amount of ROS generated in A549 cells would be, as the treating BLM concentration increase, was confirmed by DCF-DA. Here, the concentration of BLM and the amount of ROS production were proportional (Figure 5A). Then, how this interaction affects fibrosis induction was confirmed through western blot. As the concentration of treating BLM increased, NOX2 increased, and E-cadherin decreased while a-SMA increased (Figure 5B, C). This was reaffirmed through immunofluorescence (Figure 5D).



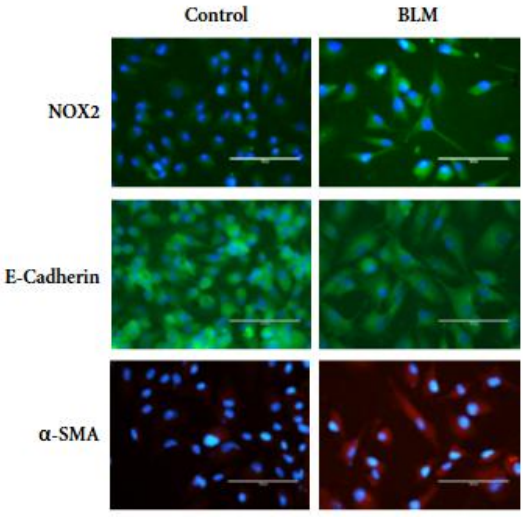


Figure 5. BLM increased NOX2 expression, resulting in EMT and fibrosis in A549 cells.

(A) The concentration of BLM and the amount of ROS production were proportional, confirmed by DCF-DA. **(B, C)** In western blot, as the concentration of treating BLM increased, NOX2 increased, and E-cadherin decreased while α-SMA increased. **(D)** The same trend was reaffirmed through immunofluorescence; while E-cadherin decreased under BLM treatment, α-SMA increased.

5. **By decreasing ROS, TMF and catechol attenuate both Smad and non-Smad signaling pathway, resulting in inhibition of EMT and fibrosis.**

As previously confirmed, *in vitro* fibrosis was induced by BLM, and ROS production and NOXs' expression were increased in that process. Then I investigated whether TMF and catechol are also effective against these BLM-induced *in vitro* fibrosis.

1)

Under both 24 and 48 hours of BLM treatment, the expression of NOX2 increased, which would have led to an increase in ROS generation, as seen in Figure 5A earlier. This increased ROS seemed to have stimulated not only TGF-β1 generation but also both the Smad and non-Smad signaling pathways which are the downstream of TGF-β1. Here, TMF and catechol had a great effect on alleviating those stimulated pathways. This attenuating effect led to inhibition of EMT and of the expression of fibrotic protein, which can be confirmed in the western blot results of BLM 48 hour treatment conditions.

2)

Sirius Red staining assay was conducted as an analysis of collagen, another fibrosis specific marker. BLM or TMF/catechol was treated for 5 days for two groups with different number of A549 cells. The cells were stained with Sirius Red and then observed under a microscope (Figure 6E). While BLM increased collagen production, TMF and catechol

decreased it. This was the same with different cell numbers. Figure 6F is a graph that quantifies red intensity in the microscopic pictures of Figure 6E.

3)

The degree of EMT and the resulting cell mobility when treated with BLM or TMF/catechol, were observed by Transwell migration analysis (Figure 6G). When BLM was treated for 72 hours, EMT of cells progressed and their migration increased, and the degree of migration was reduced by TMF and catechol. This was similar to the results under TGF-β1 treatment in Figure 3K and L. Figure 6H represents the relative value of the number of invasive cells in each plate.

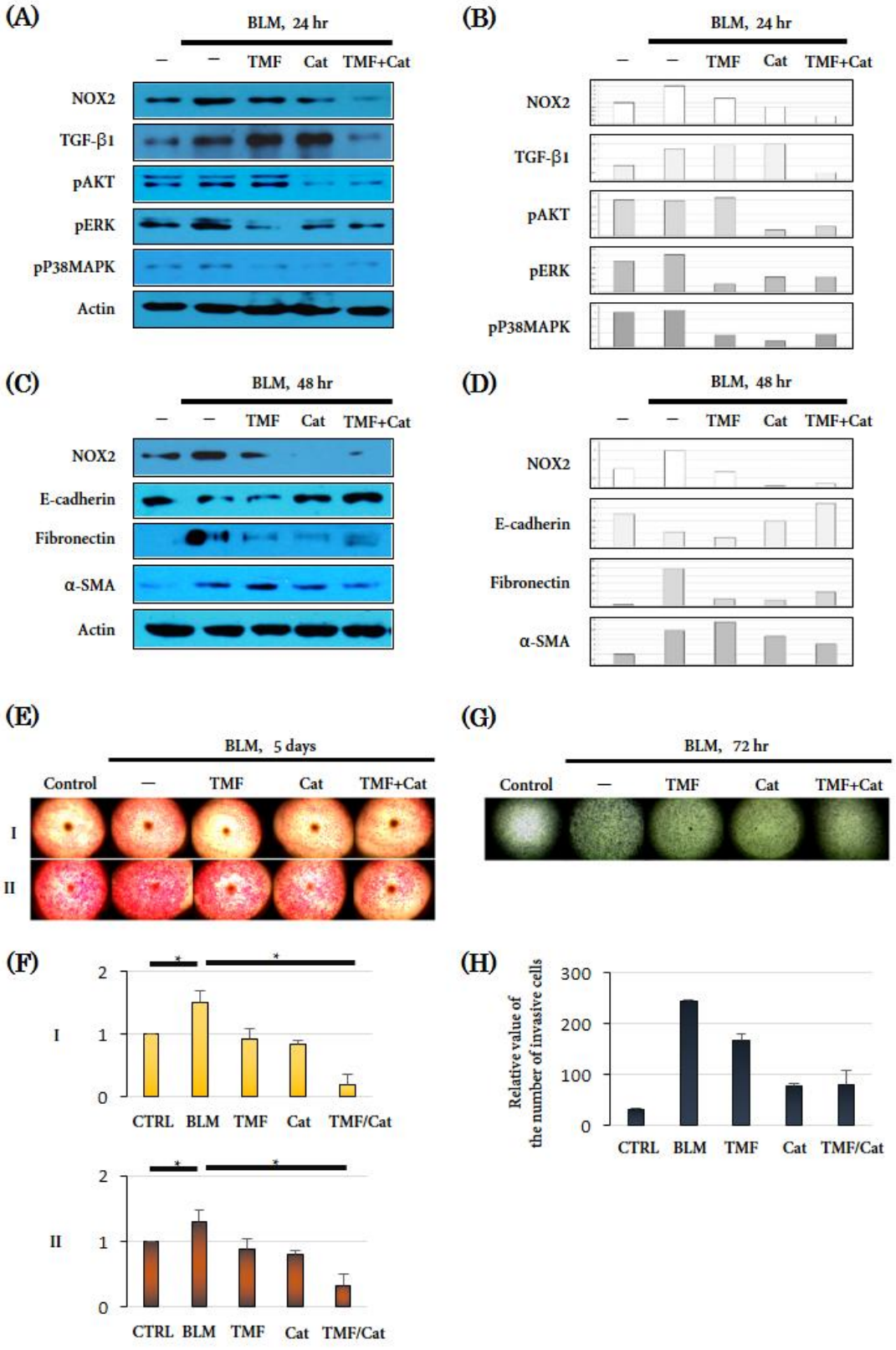


Figure 6. With decrease of NOX2, TMF and catechol attenuate both Smad and non-Smad signaling pathway, resulting in inhibition of EMT and BLM-induced fibrosis.

Under both 24 and 48 hours of BLM treatment, the expression of NOX2 increased, which would have led to an increase in ROS generation, as seen in Figure 5A earlier. **(A)** This increased ROS seemed to have stimulated both the Smad and non-Smad signaling pathways. Here, TMF and catechol had a great effect on alleviating those stimulated pathways. **(C)** This attenuating effect led to inhibition of EMT and of fibrosis, which can be confirmed in the western blot results. **(B)** and **(D)** shows the relatively quantified graphs of (A) and (C) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their GAPDH expression. **(E)** Sirius Red staining assay was conducted as an analysis of collagen, another fibrosis specific marker. While BLM increased collagen production, TMF and catechol decreased it. This was the same with different cell numbers. **(F)** is a graph that quantifies red intensity in the microscopic pictures of (E). **(G)** The degree of EMT and the resulting cell mobility when treated with BLM or TMF/catechol, were observed by Transwell migration analysis. When BLM was treated for 72 hours, EMT of cells progressed and their migration increased, and the degree of migration was reduced by TMF and catechol. **(H)** represents the relative value of the number of invasive cells in each plate.

6. TMF and catechol has antifibrotic effect in *in vivo* – BLM-induced pulmonary fibrosis in mouse lung.

It was proved in the previous experiments that TMF and catechol have inhibitory effects on BLM-induced *in vitro* fibrosis. Then, it was investigated whether those were still effective in the *in vivo* fibrosis induced by BLM. Eight C57BKL/6J male mice per group were set to inject BLM and TMF/cat for 9 weeks. After sacrifice, lung tissues and serum were taken and the following experiments were conducted.

1)

First, by performing H&E staining and Mason's trichrome staining using the tissue slides of mouse lung injected with BLM, the degree of collagen deposition was observed, and through this, whether pulmonary fibrosis was induced was confirmed. Looking at the results of each staining (Figure. 7A, B), it can be seen that the pulmonary fibrosis was significantly progressed by BLM. And when TMF or catechol was injected together with the same BLM injecting conditions, it was observed that the degree of collagen deposition was remarkably reduced. Figure 7C shows the ashcroft score as a graph, which was assigned based on the two types of staining pictures, and the effect of inhibiting fibrosis of TMF and catechol can be confirmed numerically.

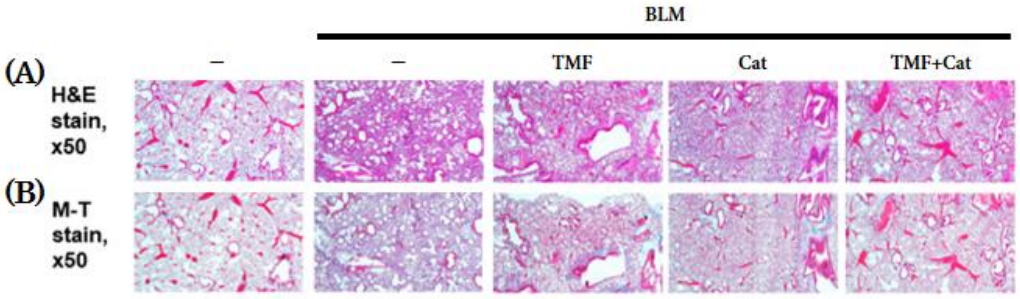
2)

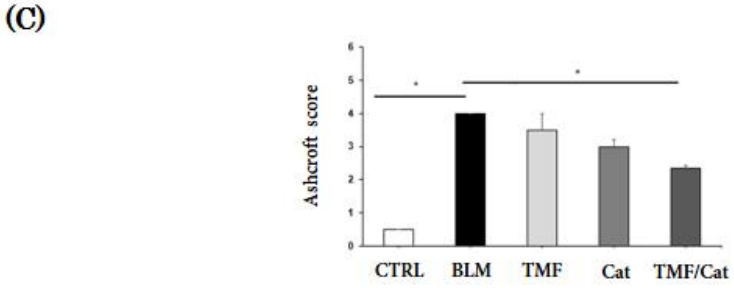
The effect of TMF and catechol was also confirmed through the results of western blot, after homogenizing the lung tissue of the mouse injected

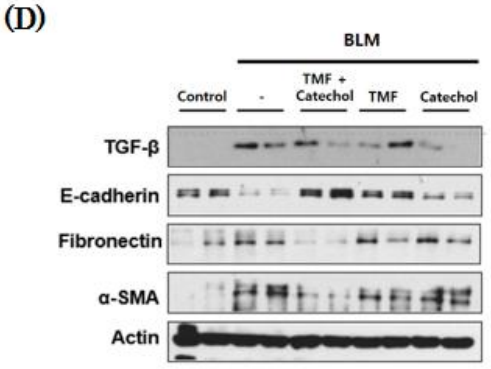
with BLM (Figure. 7D, E). TGF-β1 was produced in mouse lung by BLM, and EMT was progressed accordingly, and the expression of fibrotic protein increased as a result. Here, TMF and catechol showed effects of reducing the expression of TGF-β1, E-cadherin, Fibronectin, and a-SMA.

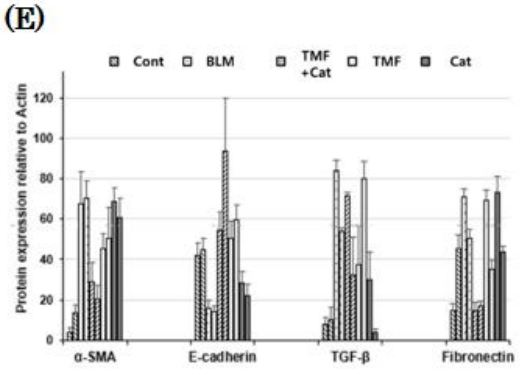
3)

In particular, the amount of TGF-β1 in mice serum was directly investigated by ELISA, and Figure 7F shows the level as a graph. As is also proven here, TMF and catechol also serve to reduce the generation of TGF-β1 itself.









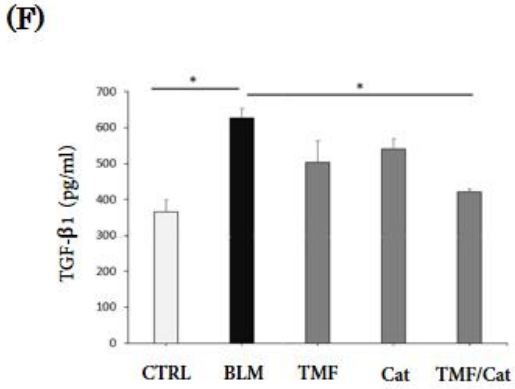


Figure 7. In *in vivo* — BLM-induced pulmonary fibrosis in mouse lung, TMF and catechol showed antifibrotic effects.

(A, B) Representative images of pulmonary tissues stained with hematoxylin and eosin (H&E) or Masson's trichrome staining were shown. It can be seen that the pulmonary fibrosis was significantly progressed by BLM. And when TMF or catechol was injected together with the same BLM injecting conditions, it was observed that the degree of collagen deposition was remarkably reduced. **(C)** shows the ashcroft score as a graph, which was assigned based on the two types of staining pictures, and the effect of inhibiting fibrosis of TMF and catechol can be confirmed numerically. **(D)** The effect of TMF and catechol was also confirmed through the results of western blot, after homogenizing the lung tissue. **(E)** shows the relatively quantified graphs of (D) results if the value of DMEM(2% FBS)-treated group is 1, with all groups considering their actin expression. **(F)** In particular, the amount of TGF-β1 was directly investigated, showing the level as a graph.

7. The antifibrotic effect of TMF and catechol is also confirmed in the reduction of inflammation accompanied by the fibrosis process.

Fibrosis is inevitably accompanied by inflammation in the mechanism of occurrence, and thus the degree of inhibition of fibrosis can be confirmed by the degree of inhibition of inflammation. Here, the number of inflammaotry cells and the level of inflammatory cytokines were investigated to check the efficacy of TMF and catechol.

1)

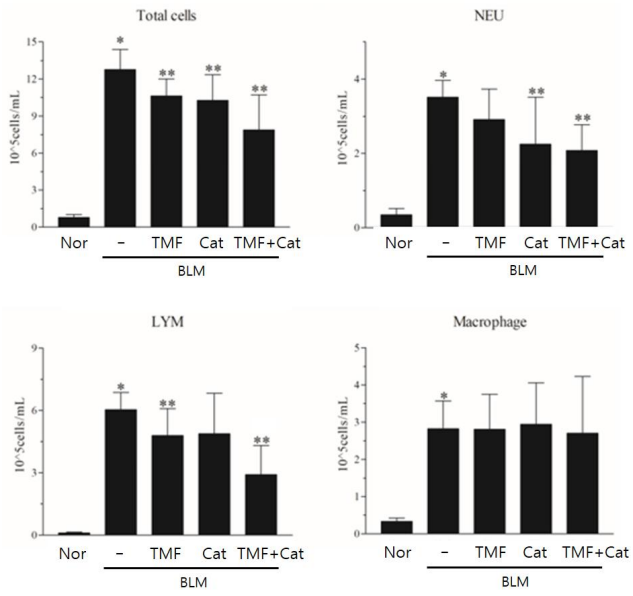
Alterations in Bronchoalveolar lavage (BAL) fluid (called BALF) and cells reflect pathological changes in the lung parenchyma. In the total cell count of BALF analysis, which is largely occupied by inflammatory cells present in alveolar space, especially macrophages, lymphocytes (LYM) and neutrophils (NEU), BLM significantly increased the number (Figure 8A). In addition, recognition of a predominantly inflammatory cellular pattern such as increased LYM or NEU in the BAL differential cell profile frequently helps us associate it with some interstitial lung diseases, including IPF. TMF and catechol increased the ratio of macrophages in total cells, while the ratio of LYM and NEU were decreased, indicating that TMF and catechol alleviated inflammation.

2)

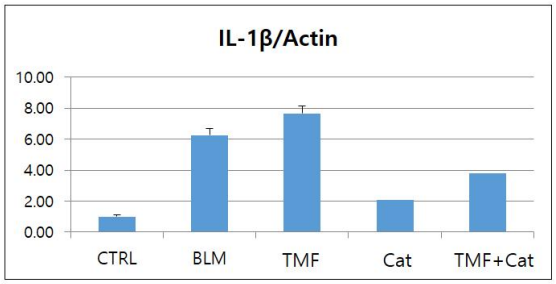
Not only the number of Inflammatory cells but also the expression level of proinflammatory cytokines secreted by them was confirmed, through quantitative RT-PCR. Figure 8B shows the normalized fold expression of

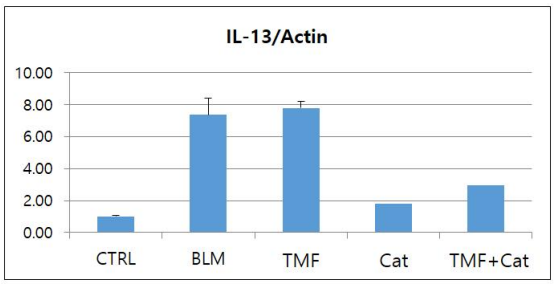
IL-1b, IL-13 and TNF-a in BLM-challenged lungs compared with control lungs. BLM significantly increased the expression level of those proinflammatory cytokines while TMF and catechol reduced the level.

(A)



(B)





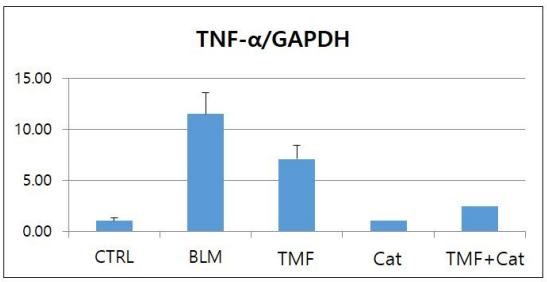


Figure 8. TMF and catechol reduced the number of inflammatory cells and the level of inflammatory cytokines.

(A) In the total cell count of BALF analysis, BLM significantly increased the number. Meanwhile, TMF and catechol increased the ratio of macrophages in total cells, as the ratio of LYM and NEU were decreased, indicating that TMF and catechol alleviated inflammation. **(B)** shows the normalized fold expression of IL-1b, IL-13 and TNF-a in BLM-challenged lungs compared with control lungs, through RT-PCR. BLM significantly increased the expression level of those proinflammatory cytokines while TMF and catechol reduced the level.

Ⅳ. DISCUSSION

Increased evidence indicates that production of ROS and oxidative stress are interlinked with production and activation of various growth factors and cytokines, which are crucial for fibrosis development and persistence. TGF-β1 was shown to promote ROS formation mainly via induction of NOX4 expression and hence activity in various types of cells. The ROS produced in response to TGF-β1 are able to activate various signaling pathways, thereby influencing gene transcription networks downstream from TGF-β1. Accordingly, numerous studies are interested in the possibility that ROS scavengers or NOX inhibitors may be used to treat IPF. For example, N-acetylcysteine (NAC) has been suggested as a beneficial treatment for IPF. The primary role of NAC is associated with its antioxidant and anti-inflammatory activity, which favors the maintenance of a cellular redox imbalance. For this reason, its therapeutic potential concerns a series of diseases that link oxidative stress to its etiology and progression. However, according to the clinical trials that have emerged in previous years, the efficacy of NAC therapy for IPF remains controversial. While certain studies have suggested that NAC therapy provides a benefit for patients with IPF and slows the deterioration of lung function, particularly the decline in forced vital capacity (FVC), others have indicated no benefit of this treatment for IPF.

It is speculated that TMF and catechol in this study may play the role of ROS scavenging, like NAC, considering their chemical structures, and indeed, many studies have been conducted on their role as ROS scavengers. In this study, the effects of TMF and catechol as fibrosis

inhibitors were explored and demonstrated in detail in both *in vitro* and *in vivo*.

Detailed and accurate exploration of their mechanism of action is needed, though. If they act as actual ROS scavengers, it is also necessary to explore where the action takes place. One guess is that TMF and catechol inhibit activation of various growth factor receptors. ROS regulates their kinase activity by engaging in sulfenylation, resulting in the change of the degree of their phosphorylation. If ROS is reduced by TMF and catechol, it may inhibit various growth factor signaling pathways within cells. Of course, it should be checked whether such an effect is actually effective in inhibiting fibrosis, and whether the degree of sulfenylation actually affects the process. In addition, if the difference in how it works with NAC is explored, it will bring about meaningful discussion of future fibrosis medicine candidates.

Ⅴ. CONCLUSION

Therapeutical effects of TMF and catechol against pulmonary fibrosis were investigated in this study (Figure 9). The effects were observed by both *in vitro* (TGF-β1, EGF and BLM-induced) and *in vivo* (BLM-induced) experiments. Herein the results suggest that TMF and catechol alone or their co-treatment significantly decrease the expression of mesenchymal cell specific markers, fibrotic proteins and inflammatory cytokines. Those overall decrease eventually result in attenuation of fibrosis. How these chemicals work should be studied and it will propose a potential medicine candidate for IPF.

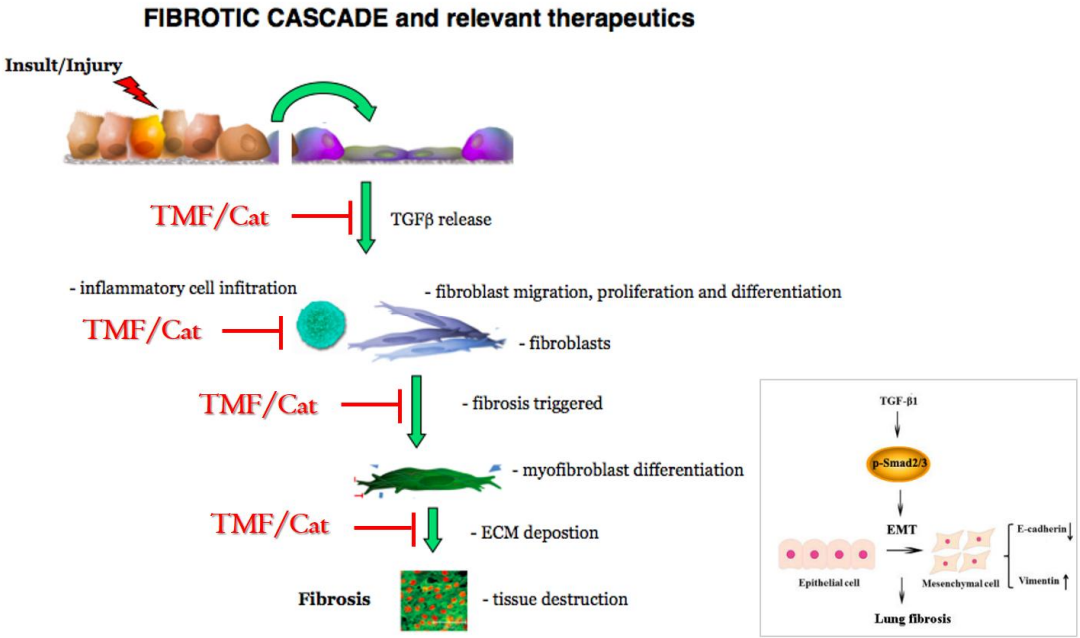


Figure 9. Overview of this study.

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국문 초록

Part 1. ROS affects EGFR phosphorylation, resulting in an acceleration of pulmonary fibrosis - *in vitro* analysis

특발성 폐섬유증 (Idiopathic pulmonary fibrosis, IPF)은 폐포 손상에 대한 점진적이며 잘못 조절된 반응으로, 과도한 세포외기질 (Extracellular matrix, ECM) 생성을 초래한다. IPF는 일반적으로 현재의 약리학적 방법에 의해 치료될 수 없어 환자들에게서 높은 사망률을 보인다.

그러나, IPF가 발생하는 분자생물학적 경로는 여전히 불분명하다. 그 경로를 규명해내는 것은 효과적인 항섬유화 치료 전략을 개발하는 데 기여할 것이다.

본 연구는 섬유화를 가속화 하는 데 있어 표피 성장인자 수용체 (Epidermal growth factor receptor, EGFR)가 하는 역할에 초점을 맞추었다. 인간 폐포상피세포인 A549에 EGF 혹은 종양증식인자 (TGF)-β1을 처리함으로써, 활성산소종(ROS)과 EGFR의 인산화가 이루는 분자축을 조사하였다. 이 시험관내 접근법은, ROS에 의해 촉진된 인산화의 결과로 EGFR의 활성화가 증가하면, 이는 섬유화를 가속화 할 수 있음을 시사했다.

또한 세포 내 ROS를 소거하거나 그것의 주 공급원인 NADPH 산화효소 (NOX)의 활성을 억제함으로써, EGFR의 인산화뿐만 아니라 섬유화 관련 인자들의 발현도 감소되는 것을 확인할 수 있었다. 또한 우리 실험실에서 항산화물질의 후보로 새로 시험하고 있는 물질들을 사용하여 EGFR 인산화가 저해되는 결과를 재확인하였다.

Part 2. TMF/catechol co-administration can be a therapeutic candidate for bleomycin-induced pulmonary fibrosis, by inhibiting EMT.

IPF는 평균 생존기간이 3 년에 불과한 치명적인 폐질환으로 여전히 난치병이다.

IPF는 활성화된 근섬유아세포에 의한 비정상적인 ECM 증착을 특징으로 한다. 그러한 근섬유아세포가 어디에서 유래되는지 수년 동안 논의되어왔으며, 상피-간엽전이 (EMT)가 근섬유아세포 활성화의 메커니즘 중 하나로 주목받고 있다.

4',6,7-트리메톡시이소플라본 (4',6,7-trimethoxyisoflavone, TMF)과 카테콜 (Catechol)이 EMT 및 섬유화에 미치는 영향을 확인하기 위해, 시험관내 TGF-β1에 의해 유도된 섬유화 모델과 생체내 블레오마이신(Bleomycin, BLM)에 의해 유도된 섬유화 모델을 사용했다. 그 결과 TMF/Catechol의 공동 투여는, TGF-β1의 Smad 및 non-Smad 신호 경로를 저해함으로써 EMT 및 ECM 축적을 억제하여, 유도되었던 폐섬유화를 완화하였다. 또한 BLM을 주사한 생쥐의 폐에서 유의미하게 증가되었던 염증성 사이토카인(IL-1β, IL-13, TNF-α)의 수치가, TMF/Catechol 공동치료에 의해 감소되었다.

위와 같은 연구 결과는 TMF/Catechol을 사용한 공동치료가 폐섬유화에 대한 잠재적 치료 후보일 수 있음을 시사한다.

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