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Original Article

Antifibrotic effects of Quercetin on TGF-β1-induced vocal fold fibroblasts

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Abstract: Objective: To investigate the effects of Quercetin on vocal fold fibroblasts induced by TGF-β1 and vocal fold injury. Methods: The effects of Quercetin on collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN) expressions in transforming growth factor-β1 (TGF-β1)-induced human vocal fold fibroblasts were examined by the quantitative reverse transcription-polymerase chain reaction and the enzyme-linked immunosorbent assay. The Cell Counting Kit-8 was used to assess the influence of Quercetin on cell proliferation. A rat vocal fold injury model was developed. These rats were divided into a control group, a model group, and a Quercetin group. Pathorphological observations from Hematoxylin-eosin staining, the density of fibroblasts, and hypertrophic index were compared among the three groups. Results: Compared with cells untreated with TGF-β1, the mRNA and protein expression levels of COL-I, COL-II, and FN and proliferation ability in TGF-β1-induced human vocal fold fibroblasts were increased (all $P<0.05$). Compared with human vocal fold fibroblasts induced by TGF-β1, the mRNA and protein expression levels of COL-I, COL-II, and FN and proliferation ability in TGF-β1-induced human vocal fold fibroblasts following Quercetin stimulation were decreased (all $P<0.05$). There were no statistical differences between human vocal fold fibroblasts treated with or without Quercetin regarding cell proliferation and COL-I, COL-II, and FN expressions. Compared with the model group, Quercetin suppressed the fibrosis of the vocal fold following injury and the protein levels of COL-I, COL-II, and FN in tissue samples (all $P<0.001$). The density of fibroblasts and hypertrophic index in the vocal fold scar from the Quercetin group were lower than those in the model group (all $P<0.05$). Conclusions: Quercetin inhibited TGF-β1-induced fibrotic changes and proliferation in human vocal fold fibroblasts and had an antifibrotic effect in vocal fold after injuries.

Keywords: Vocal fold injury, animal model, Quercetin, fibrosis

Introduction

The vocal fold mucosa plays an important role in phonation. It is in the larynx and covered by epithelial cells and lamina propria consisted of an extracellular matrix (ECM) network of interstitial proteins, fibrous, and glycans [1, 2]. It has been shown that the native lamina propria is mainly populated by fibroblast cells. The pathological injuries of the vocal fold mucosa resulted in fibrosis formation in the lamina propria [3]. Vocal fold fibrosis is demonstrated to be a major cause for intractable dysphonia which leads to occupational and social limitations, reduces life quality, and increases healthcare expenses [4]. A number of treatment options have been developed for vocal fold injury. New biomaterials and growth factor treatments hold clinical promise. It is associated with unsustained benefits and risk of iatrogenic damage [5, 6]. Prevention and treatment of vocal fold fibrosis has become a target that has attracted attention for vocal fold injury.

There are a variety of reasons for the etiology of vocal fold fibrosis. It has been demonstrated that during the period of tissue repair, the sequential changes in density and morphology of fibroblasts and ECM composition occur in the vocal fold mucosa [7, 8]. An accumulation of fibrous composition enhances the tissues' stiffness and reduces the pliability, necessary for the vibration function [9]. Transforming growth factors β (TGF-β) is known to be the most repre-

sentative factor in the development and progression of fibrosis and hypertrophic scar [10, 11]. TGF-β1 has the strongest activity among TGF-β members. The abnormally increased TGF-β1 expression and intracellular signal transductions are positively correlated with the phenotypic transition of fibroblasts [12]. Many studies have shown that down-regulation of TGF-β1 expression decreases or inhibits the process of fibrosis [13]. TGF-β1 plays an important role in the development of vocal fold fibrosis and is a therapeutic target in pathologic fibrogenesis.

Quercetin is a kind of flavonoids compound, which widely exists in various materials such as vegetables, fruits, and tea [14]. Quercetin has been reported to have immunoregulatory, anti-inflammatory, and antioxidative effects, and inhibit the angiogenesis [15]. The underlying mechanisms are unknown. Quercetin has been shown to prevent myocardial infarction adverse remodeling in rats by suppressing TGF-β1 signaling [16]. Wu et al. reported that Quercetin inhibited hepatic fibrosis by decreasing autophagy and inhibiting activation of hepatic stellate cells through TGF-β1 pathways [17]. The roles of Quercetin in vocal fold fibrosis and its molecular mechanism have not been fully elucidated.

We conducted an in vitro experiment using human vocal fold fibroblasts and an in vivo study using a rat model of vocal fold injury, and hypothesized that Quercetin influences inhibiting vocal fold fibrosis. Collage type I (COL-I), Collage type III (COL-III), and fibronectin (FN) were exploited as fibrotic markers in this research. The results of this study provided experimental evidence for the treatment of vocal fold fibrosis.

Materials and methods

Cell culture

This study was in accordance with the rules and regulations of the laboratory and approved by The Institutional Animal Care and Use Committee of Shandong University (Approval No. 2020-138). Human vocal fold fibroblasts (American Type Culture Collection, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) and maintained in an incubator with the condition of 5% carbon dioxide at 37°C. Before the experiment, the cells were cultured in DMEM containing 0.5% FBS for 12 h. Afterwards, the cells were treated with 10 ng/mL of TGF-β1 and/or 250 μmol/L Quercetin.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) with incubation for 48 h was used to detect the proliferation of human vocal fold fibroblasts according to the recommended protocol. Human vocal fold fibroblasts were inoculated into 96-well plates at a density of 4×10^3 cells/well. CCK-8 solutions were added to each well. The optical density for each well was measured with a microplate reader at 450 nm (Bio-Rad Laboratories, Inc. USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

According to a previous study [18], the total RNA of human vocal fold fibroblasts was extracted from each group after being treated by the Trizol method (Invitrogen). The reverse transcription kit was used to synthesize the first strand cDNA (Takara, Japan) in accordance with the instructions of the manufacturer. The mRNA was quantitatively analyzed by TB SYBR Premix Ex Taq (Takara, Japan). The relative gene expression was analyzed by $2^{-\Delta\Delta}$CT method. Primers applied for qRT-PCR amplification are detailed in **Table 1**.

Enzyme-linked immunosorbent assay (ELISA)

The tissue samples of the vocal fold from rat models were homogenized. Human vocal fold fibroblasts were cultured and treated with TGF-β1 and/or Quercetin. Supernatant was collected and centrifuged at 3500 rpm for 5 min. The contents of COL-I (R&D systems, USA), COL-III (R&D systems, USA), and FN (R&D systems, USA) were analyzed using corresponding ELISA Kits in accordance with the instructions of the manufacturer.

Rat model of vocal fold injury

A total of 30 young male Sprague-Dawley rats with the average body weight of 290±10 g were employed in this study. Their ages were 14 to 15 weeks. The rats were obtained from The Experimental Animal Center in our hospital. The

Table 1. Primer sequences for RT-PCR

Gene	Primer sequence
COL-I	Forward: 5'-GTGCTAAAGGTGCCAATGGT-3'
	Reverse: 5'-GTGGGGAATGGCAAGCAAAA-3'
COL-III	Forward: 5'-CCAGGAGCTAACGGTCTCAG-3'
	Reverse: 5'-CAGGGTTTCCATCTCTTCCA-3'
FN	Forward: 5'-TGGAACTTCTACCAGTGCGAC-3'
	Reverse: 5'-TGTCTTCCCATCATCGTAACAC-3'
β-actin	Forward: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'
	Reverse: 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'

Note: Collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN).

housing, breeding, and animal experiments were approved by the Institutional Animal Care and Use Committee of our hospital (approval No. 2021-174) and were in accordance with the rules and regulations on the use of laboratory animals. According to the protocol described in previous studies [10], the unilateral vocal fold injury model was established. Ketamine hydrochloride (90 mg/kg) was intraperitoneally injected for the anesthetization in each rat. The rat was kept in a near-vertical position on an operative platform. The larynx was exposed with the help of an endoscope. The left vocal fold was damaged by separating the lamina propria using a 25-gauge needle at the lateral edge of the left vocal fold, and removed using micro-forceps. The oozing of blood around the glottis was soaked up, and an unobstructed respiratory tract was maintained. These rats were randomized into the following three groups: control group, model group, and Quercetin group. Intragastrical administration of 25 mg/kg Quercetin was performed once per day for one month in the Quercetin group. An equal volume of normal saline was administered to rats from the control group and the model group. At the end of the study period, all the rats were sacrificed under CO_2 anesthesia. Following the sacrifice, the larynx was harvested and prepared for additional research.

Hematoxylin-eosin (HE) staining

The vocal fold tissues were fixed in 4% neutral formalin and embedded in paraffin. Sections of 5 μm from each paraffin block were stained with hematoxylin and eosin described by previous studies [19]. The sections were immersed in xylene and alcohol. The sections were stained with hematoxylin for 2 minutes and eosin for 30 seconds. They were immersed in alcohol and xylene again. The slides were mounted by the synthetic resin.

Under a light microscope, the number of fibroblasts per unit area was examined in five high power fields (×200) of vocal fold scar from each group. The hypertrophic index was calculated in the three groups. The hypertrophic index was defined as the ratio of vocal fold scar thickness to normal vocal thickness.

Statistical analysis

All statistical data in this study was analyzed by SPSS 22.0 software. The measurement data were presented by mean ± standard deviation (SD). The independent sample t-test was exploited for the comparison between the two groups. One-way ANOVA and post hoc comparison with Bonferroni were performed among the three groups. $P<0.05$ indicated the statistical significance.

Results

TGF-β1 induced the levels of COL-I, COL-II, FN, and cell proliferation in human vocal fold fibroblasts

To detect TGF-β1-induced fibrosis of vocal fold fibroblasts, the levels of COL-I, COL-II, and FN were evaluated in TGF-β1-induced human vocal fold fibroblasts. Human vocal fold fibroblasts were stimulated with TGF-β1 for 24 h to induce ECM proteins. Compared with the control group, the COL-I, COL-II, and FN mRNA levels in the TGF-β1 group increased significantly (**Figure 1A-C**). ELISA results demonstrated enhanced COL-I, COL-II, and FN levels in cell supernatant (**Figure 2B-D**). The results of CCK-8 indicated that the proliferation capacity of cells in the TGF-β1 group was more than that in the control group (**Figure 2A**). These findings suggested that TGF-β1 promoted ECM proteins and proliferation capacity of human vocal fold fibroblasts, which exerted a supplementary effect in the progression of vocal fold fibrosis.

Quercetin suppressed TGF-β1-induced ECM synthesis and cell proliferation in human vocal fold fibroblasts

The effects of Quercetin on TGF-β1-induced ECM synthesis in human vocal fold fibroblasts

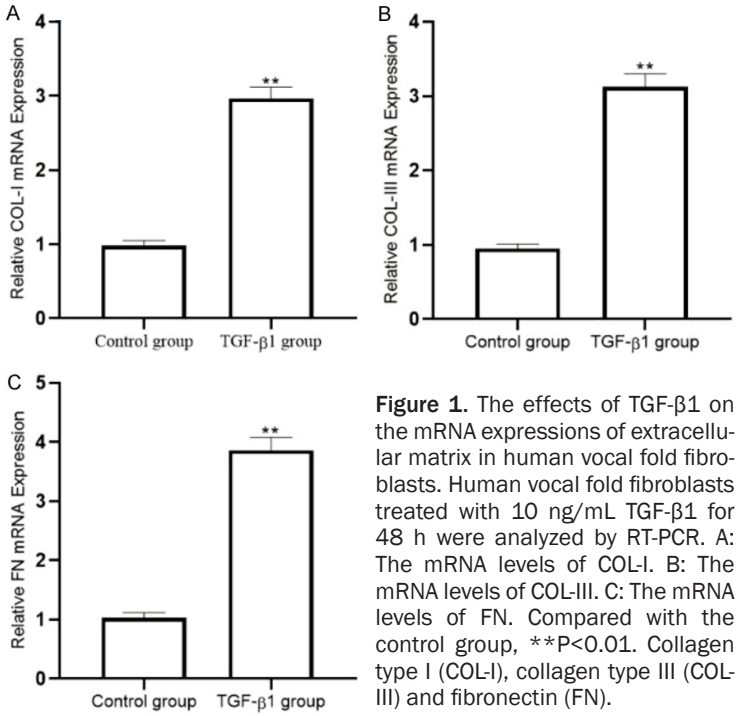

Figure 1. The effects of TGF-β1 on the mRNA expressions of extracellular matrix in human vocal fold fibroblasts. Human vocal fold fibroblasts treated with 10 ng/mL TGF-β1 for 48 h were analyzed by RT-PCR. A: The mRNA levels of COL-I. B: The mRNA levels of COL-III. C: The mRNA levels of FN. Compared with the control group, **P<0.01. Collagen type I (COL-I), collagen type III (COL-III) and fibronectin (FN).

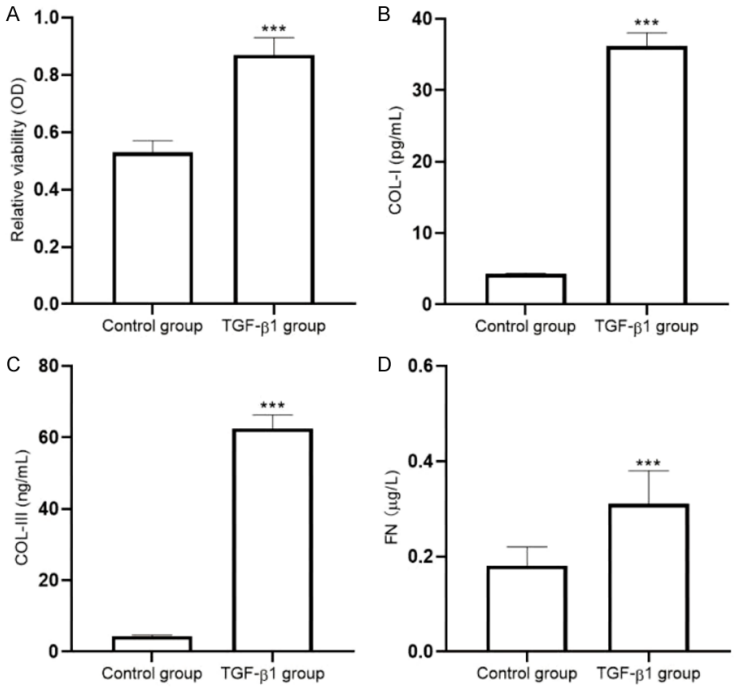

Figure 2. The effects of TGF-β1 on the proliferation and extracellular matrix expression levels in human vocal fold fibroblasts. Proliferation ability of human vocal fold fibroblasts treated by 10 ng/mL TGF-β1 was detected by CCK-8 assay. The concentration of extracellular matrix proteins was detected by ELISA assay. A: Cell proliferation. B: COL-I. C: COL-III. D: FN. Compared with the control group, ***P<0.001. Collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN).

were evaluated. The findings of qRT-PCR suggested that Quercetin decreased the mRNA levels of COL-I, COL-II, and FN in TGF-β1-induced human vocal fold fibroblasts (**Figure 3A-C**). The results of ELISA demonstrated the reduced COL-I, COL-II, and FN proteins levels in cell supernatant from TGF-β1-induced human vocal fold fibroblasts (**Figure 4B-D**). The findings of CCK-8 assay revealed that Quercetin inhibits the proliferation of TGF-β1-induced human vocal fold fibroblasts. There were differences in cell proliferation between the TGF-β1 group and the Quercetin+TGF-β1 group (**Figure 4A**). These findings suggested that Quercetin inhibited ECM synthesis and cell proliferation in TGF-β1-induced human vocal fold fibroblasts.

The effects of Quercetin on ECM synthesis and cell proliferation in human vocal fold fibroblasts

As shown in **Figures 5** and **6**, the mRNA and proteins levels of COL-I, COL-II, and FN in human vocal fold fibroblasts treated with 250 μmol/L Quercetin were similar with those in the control group. The statistical differences between the two groups were not significant. Regarding cell proliferation, the proliferation ability of human vocal fold fibroblasts treated with 250 μmol/L Quercetin were not significantly lower than that in the control group. No statistical difference was found between the two groups.

Comparison of HE staining results among the groups

The results of HE staining are in **Figure 7**. Compared with the control group, the components of

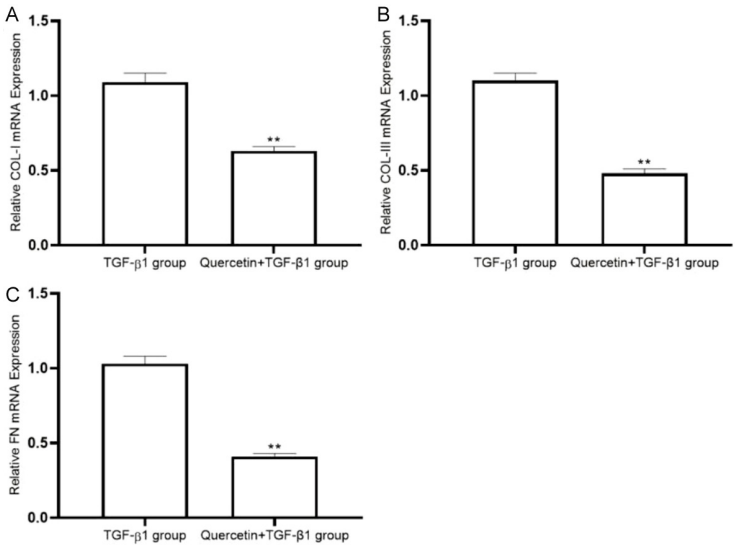

Figure 3. The effects of Quercetin on mRNA expression of extracellular matrix in TGF-β1-induced human vocal fold fibroblasts. A-C: mRNA levels of COL-I, COL-II, and FN in TGF-β1-induced human vocal fold fibroblasts treated with Quercetin for 48 h were analyzed by RT-PCR. Compared with the TGF-β1 group, **P<0.01. Collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN).

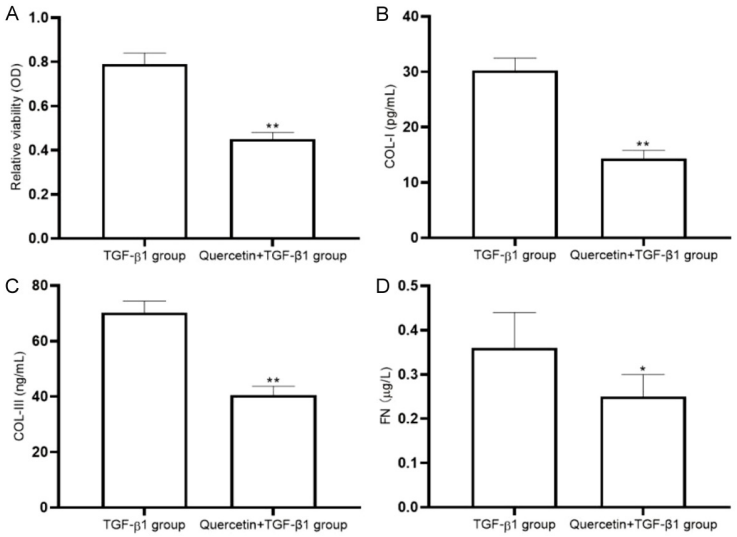

Figure 4. The effects of Quercetin on cell proliferation and concentration of extracellular matrix proteins in TGF-β1-induced human vocal fold fibroblasts. A: Proliferation ability of TGF-β1-induced human vocal fold fibroblasts after treating with Quercetin was detected by CCK-8 assay. B-D: ELISA assay was used to examine the concentrations of COL-I, COL-II, and FN in TGF-β1-induced human vocal fold fibroblasts treated with Quercetin. Compared with the TGF-β1 group, *P<0.05 and **P<0.01. Collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN).

fibrous tissues were increased. The collagen fibers with disordered arrangements were accumulated in the lamina propria of the vocal fold in the model group. Compared with the model group, the components of the fibrous tissues were decreased. The collagen fibers were slightly regularly arranged in the Quercetin group.

Comparison of hypertrophic index and fibroblast density among the three groups

As shown in **Table 2**, the hypertrophic index and fibroblast density in the model group were 2.87±0.25 and 29.16±3.04, respectively. The hypertrophic index and fibroblast density in the Quercetin group were lower than those in the model group, but higher than those in the control group.

Comparison of COL-I, COL-II, and FN protein levels among the three groups

As seen in **Figure 8**, compared with those in the control group, the protein levels of COL-I, COL-II, and FN were higher in the model group and the Quercetin group (all P<0.05). In contrast to those in the model group, the protein levels of COL-I, COL-II, and FN in the Quercetin group were decreased (P<0.001).

Discussion

Voice disorder is the most common communication dysfunction in the lifespan. Vocal fold tissue health bears the fundamental capacity for oral communication. The vocal fold fibrosis is a major reason for intractable dysphonia [20]. It is important to apply effective drugs to inhibit vocal fold fibrosis, to reduce the vibratory dysfunction and to improve the prognosis of the affected patients [8]. Disorganized composition of the ECM is the pathological basis of vocal fold scarring [21]. The excessive fibronectin deposition and the accumulation of collage deposition in the ECM are vital pathological features of vocal fold scarring [7]. It was reported that the inflammation or damages of the vocal fold tissue acti-

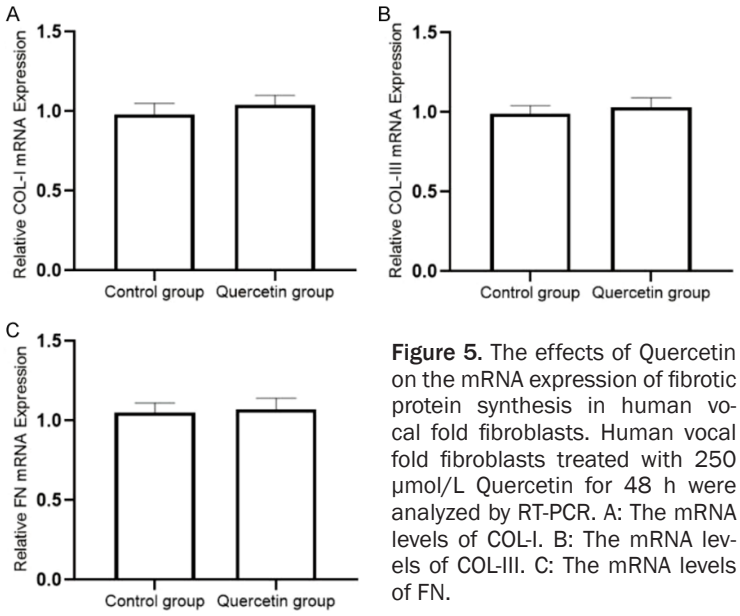

Figure 5. The effects of Quercetin on the mRNA expression of fibrotic protein synthesis in human vocal fold fibroblasts. Human vocal fold fibroblasts treated with 250 μmol/L Quercetin for 48 h were analyzed by RT-PCR. A: The mRNA levels of COL-I. B: The mRNA levels of COL-III. C: The mRNA levels of FN.

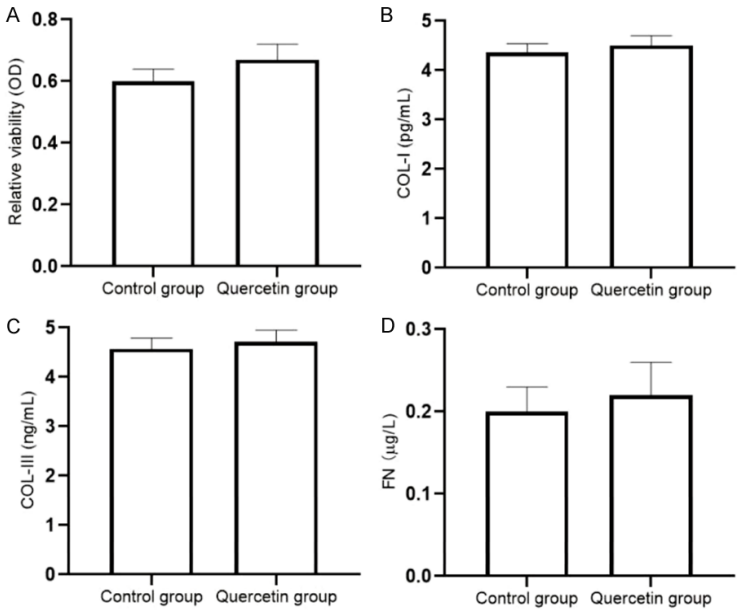

Figure 6. The effects of Quercetin on the proliferation and extracellular matrix expression levels in human vocal fold fibroblasts. Proliferation ability of human vocal fold fibroblasts treated by 250 μmol/L Quercetin was detected by CCK-8 assay. The concentrations of extracellular matrix proteins were detected by ELISA assay. A: Cell proliferation. B: COL-I levels. C: COL-III levels. D: FN levels. Collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN).

vates fibroblasts, which transform into myofibroblasts to enhance wound healing [22]. The ECM proteins including COL-I, COL-III, and FN are synthesized by activated myofibroblasts and fibroblasts. Hiwatashi et al. [23] reported that COL-III expressed in the early stage of wound healing, followed by the expression of COL-I, indicated that COL-III was more important than COL-I in the early stage of wound healing. The fibrosis response of vocal fold has been one of the research hotspots in this field. Expressions of ECM proteins play an important role in vocal fold fibrosis.

The major modulator of ECM proteins is the TGF-β superfamily, especially TGF-β1. In this study, the results showed that TGF-β1 caused an increase in the production of COL-I, COL-III, and FN in human vocal fold fibroblasts: incubation with 10 ng/mL TGF-β1 for 48 h generated an obvious increase in all three proteins. This finding is similar with that of previous research projects [12, 24]. In this study the production of COL-I, COL-III, and FN was examined in TGF-β1 induced human vocal fold fibroblasts in the absence or presence of Quercetin. In recent years, the role of TGF-β1 has been considered as a master regulator of the accumulation of ECM proteins and a key driver of fibrosis [25]. COL-I and COL-III are the major collagens whose expressions are stimulated to restore tissue integrity and tensile strength. Studies have proven that TGF-β1 was a necessary cell factor for the synthesis of these collagens following the injury of tissues [26]. Increased evidence showed that TGF-β1 activated the expressions of many proteins participating in cell-cycle control such as regulators of cyclin-dependent kinases, mitogen activated protein kinase signaling, and phosphatidylinositol 3-hydroxy kinase pathway. This enhanced the proliferation of cells [27]. Some studies revealed the proliferation of fibroblasts was induced by TGF-β in various tissues [28]. Another study showed that TGF-β had no effect on the proliferation of fibroblasts in cell

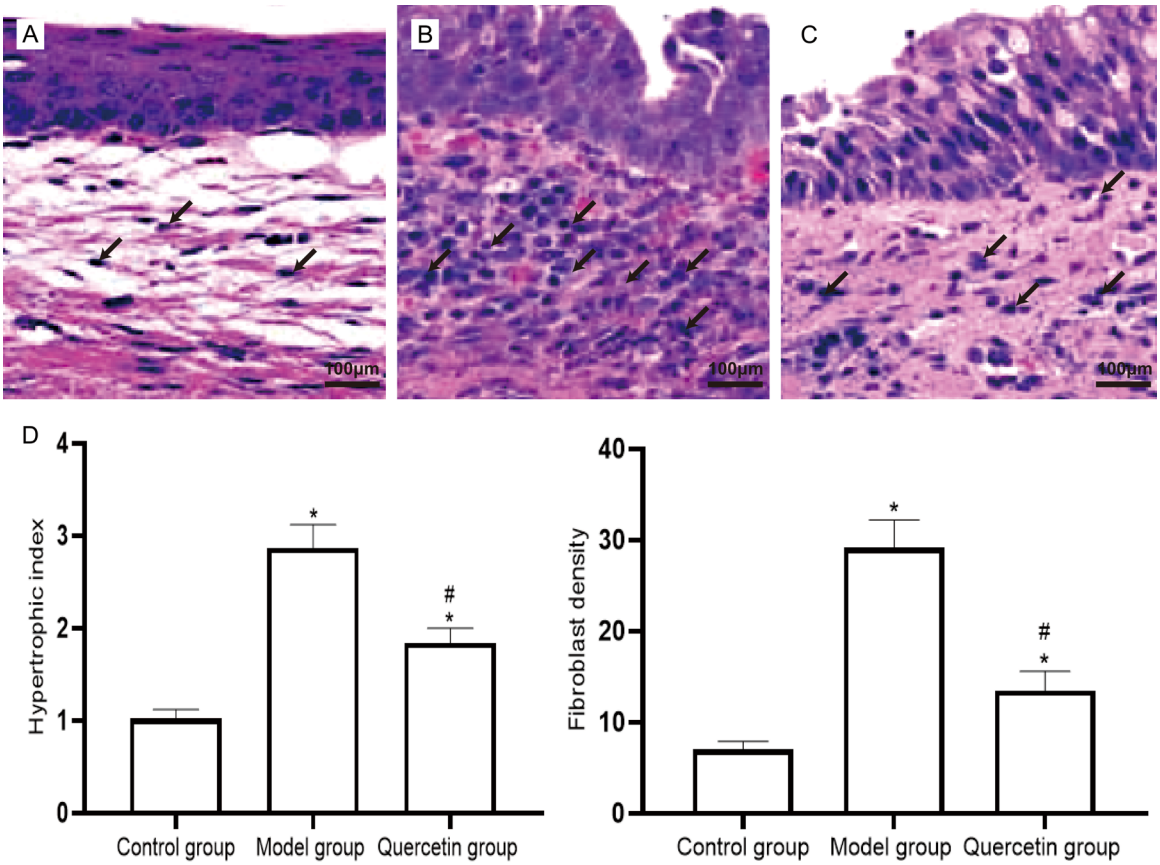

Figure 7. Hematoxylin and eosin staining in the vocal fold of rats from the three groups (×200). Arrows indicated fibroblasts. A: Control group. B: Model group. C: Quercetin group. D: Comparison of hypertrophic index and density of fibroblasts among the three groups.

Table 2. Comparison of hypertrophic index and density of fibroblasts among the three groups

Groups	Hypertrophic index	Fibroblast density
Control group	1.02±0.10	7.05±0.86
Model group	2.87±0.25*	29.16±3.04*
Quercetin group	1.84±0.16*,#	13.47±2.15*,#

Note: Compared with the control group, *P<0.05; Compared with the model group, #P<0.05.

culture [29]. These conflicting findings on the role of TGF-β in promoting the proliferation of fibroblasts can be resolved by understanding the co-stimulatory activation of signaling in response to TGF-β. In this study, the results showed TGF-β1-induced proliferation of human vocal fold fibroblasts, indicating that the microenvironment with TGF-β1 stimulating proliferation of fibroblasts determined the response of proliferation in fibrosis.

From the perspective of traditional Chinese medicine, vocal fold fibrosis belongs to *qi stagnation*. Numerous studies have shown that many Chinese medicines have relieved the clinical symptoms caused by organ fibrosis [30]. Quercetin, as a primary representative of flavonoids, has a variety of biological activities. Previous studies have shown that Quercetin had an inhibition effect on organ fibrosis such as renal fibrosis, pulmonary fibrosis, and myocardial fibrosis through affecting various growth factors and cell signaling pathways [31, 32]. Wu et al. found that Quercetin has reduced liver fibrosis induced by bile duct ligation and carbon tetrachloride in mice through inhibiting the TGF-β1/Smad signaling pathway and the activation of hepatic stellate cells [17]. Nakamura et al. reported that Quercetin inhibits idiopathic pulmonary fibrosis by regulating TGF-β-induced collagen production in normal human lung fibroblasts [33]. In this study, we investigated the effects of Quercetin on vocal fold fibrosis.

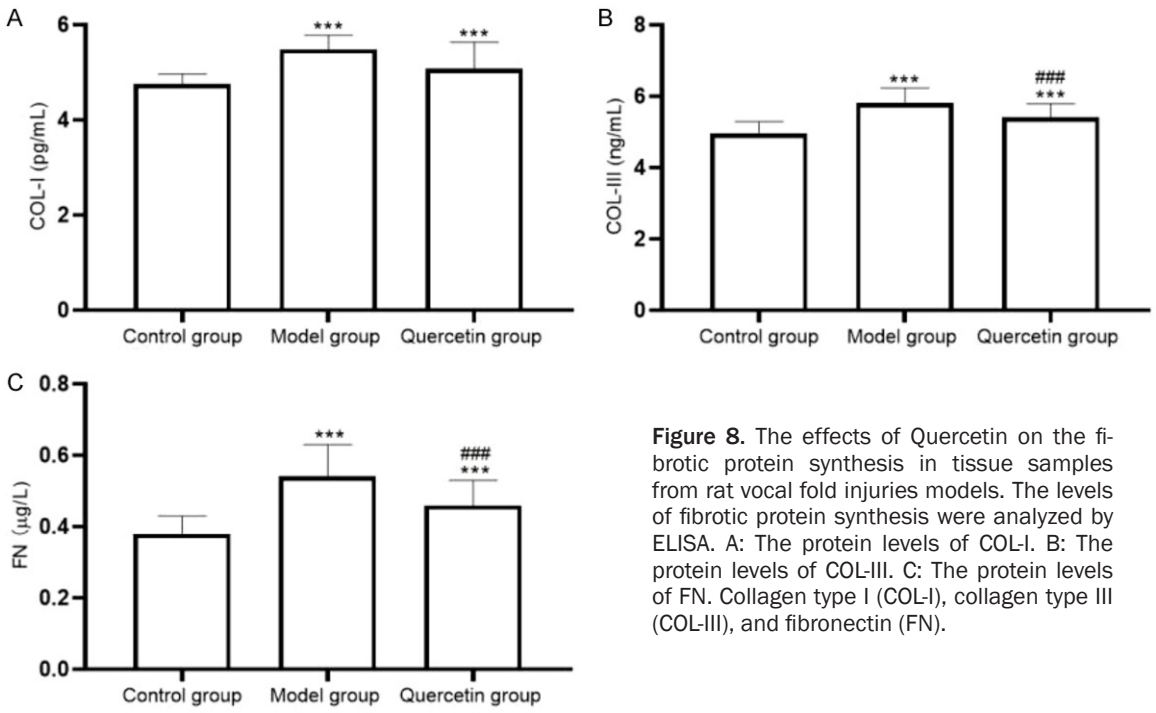

Figure 8. The effects of Quercetin on the fibrotic protein synthesis in tissue samples from rat vocal fold injuries models. The levels of fibrotic protein synthesis were analyzed by ELISA. A: The protein levels of COL-I. B: The protein levels of COL-III. C: The protein levels of FN. Collagen type I (COL-I), collagen type III (COL-III), and fibronectin (FN).

The mRNA and protein expressions of COL-I, COL-III, and FN in TGF-β1-induced human vocal fold fibroblasts after Quercetin intervention were reduced. The results of the rat vocal fold injury model showed that the fibrosis of vocal fold following injury was improved after Quercetin intervention. This indicated that the therapeutic effect of Quercetin against vocal fold scarring was achieved by inhibiting the activation of fibroblasts. For the treatment of vocal fold injury, Quercetin mainly inhibited the excessive deposition of ECM to improve vocal fold fibrosis. Its mechanism of action inhibits the activation of the TGF-β1 pathway. This was consistent with previous studies on Quercetin reversing fibrosis in other tissues [34, 35].

This study had some limitations that should be noted. Our study could not fully replicate the vocal fold scarring in humans. The mechanisms of signal transduction system for Quercetin intervention were not further evaluated. Additional long-term and in vivo human studies are needed to confirm our findings and to support Quercetin as a therapeutic drug targeting TGF-β1 signaling in vocal fold fibrosis.

In conclusion, TGF-β1 treatment induced the proliferation of cells and stimulated the expression of fibrotic ECM proteins in human vocal fold fibroblasts. Quercetin intervention suppressed the proliferation of cells and decreased the protein expressions of COL-I, COL-III, and FN in TGF-β1 induced human vocal fold fibroblasts and the vocal fold fibrosis after injuries in animal models. Our findings indicated implications for TGF-β1 targeted antifibrotic treatment for the scarring of vocal fold.

Disclosure of conflict of interest

None.

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