Journal of Dental Sciences 19 (2024) 2100–2105

Available online at www.sciencedirect.com

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Original Article

Effects of *lactobacillus pentosus* postbiotics on fibrotic response in arecoline-induced oral fibrogenesis

Po-Yu Yang [a,b], Chin-Chu Chen [c,d], You-Shan Tsai [c], Yi-Wen Liao [e,f], Min Yee Ng [a], Chun-Chung Huang [e], Cheng-Chia Yu [b,e*], San-Fu Hong [g]**

[a] *School of Dentistry, Chung Shan Medical University, Taichung, Taiwan*
[b] *Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan*
[c] *Biotech Research Institute, Grape King Bio Ltd., Taoyuan, Taiwan*
[d] *Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan*
[e] *Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan*
[f] *Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan*
[g] *Department of Otorhinolaryngology, Head and Neck Surgery, Changhua Christian Hospital, Changhua, Taiwan*

Received 23 March 2024; Final revision received 10 April 2024
Available online 23 April 2024

KEYWORDS
Oral submucous fibrosis;
Myofibroblasts;
GK4;
Postbiotic

Abstract *Background/purpose:* Oral submucous fibrosis (OSF), characterized by excessive collagen deposition by myofibroblasts, is often linked to Areca nuts consumption. Probiotics consumption has shown protective effects against fibrotic diseases, and recently, their metabolic byproducts, known as postbiotics, have demonstrated superior advantages over probiotics. However, studies on the therapeutic impact of postbiotics on OSF have been scarce. Therefore, this study aims to examine the effect of PostBio GK4, a postbiotic derived from *Lactobacillus pentosus GK4*, on OSF and explore its underlying mechanisms.
Materials and methods: The cytotoxicity of GK4 in normal buccal mucosal fibroblasts (BMFs) and fibrotic BMFs (fBMFs) were assessed. Following this, we evaluated the effects of GK4 on collagen contraction, migratory, and wound healing capacities in arecoline-induced fibrotic BMFs. Next, Western blotting and ELISA were employed to assess GK4's impact on fibrosis-related proteins such as COL1A1, and α-SMA, as well as on TGF-β and Smad2/3 signaling pathway.
Results: Arecoline was shown to stimulate cell migratory, contractile and wound healing

* Corresponding author. Institute of Oral Sciences, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 40201, Taiwan.
** Corresponding author. Department of Otorhinolaryngology-Head and Neck Surgery, Changhua Christian Hospital, No. 135 Nanhsiao Street, Changhua 50006, Taiwan.
E-mail addresses: ccyu@csmu.edu.tw (C.-C. Yu), 149184@cch.org.tw (S.-F. Hong).

https://doi.org/10.1016/j.jds.2024.04.009

abilities as well as the expression of α-SMA and COL1A1 in BMFs. Treatment with GK4 reduced all arecoline-induced phenomena in BMFs. Moreover, GK4 diminished the increased expression of TGF-β and Smad2/3.

Conclusion: Our findings proposed that GK4 may exert a suppressive effect on arecoline-induced myofibroblast activities via the inhibition of TGF-β and Smad2/3 signaling pathway. Therefore, GK4 holds promise as an adjunct therapeutic approach for intervening in OSF. Further in-vivo and clinical studies are warranted to validate these observations.

Introduction

Oral submucous fibrosis (OSF) is a preneoplastic condition characterized by excessive collagen deposition in the oral submucosa, resulting in progressive limitation of mouth opening and difficulty with eating and swallowing. OSF is prevalent among 10–20% of populations in South and Southeast Asia, where chewing of areca nut is a widespread habit.[1,2] Arecoline, the principal alkaloid of areca nut, is a major risk for OSF as it stimulates collagen synthesis and induces myofibroblast differentiation of buccal mucosal fibroblasts (BMFs).[3,4]

Myofibroblasts are contractile cells characterized by the expression of α-smooth muscle actin (α-SMA) that are key mediators of extracellular matrix (ECM) production and tissue remodeling during fibrosis.[5] In OSF, persistent myofibroblast activation leads to excessive collagen deposition and progressive fibrosis.[6,7] Arecoline has been shown to induce myofibroblast phenotypes in BMFs, enhancing their contractility, migration, and wound healing capacities.[7–10] Myofibroblasts also overexpress ECM components including type I collagen (COL1A), resulting in tissue stiffening and loss of flexibility.[11] Therefore, targeting myofibroblast activity represents a potential therapeutic approach for halting OSF progression.

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts.[12,13] Studies have revealed promising effects of certain probiotic strains in attenuating fibrotic diseases. For instance, *Lactobacillus rhamnosus GG* was found to alleviate liver fibrosis in a rat model by downregulating profibrogenic genes and reducing collagen deposition.[14] Additionally, *Lactobacillus plantarum LS/07* protected against liver fibrosis progression in a murine model, potentially by modulating oxidative stress and inflammation.[15] The antifibrotic effects have been attributed to immunomodulation, downregulation of profibrotic cytokines like TGF-β, and inhibition of signaling pathways driving myofibroblast activation such as Smad2/3 signaling.[16]

While probiotics have demonstrated protective effects, there has been emerging interest in their metabolic byproducts known as postbiotics. Postbiotics are bioactive compounds produced during probiotic fermentation, such as organic acids, bacteriocins, and enzymes.[17] Compared to probiotics, postbiotics offer enhanced stability across various pH, temperature, and storage conditions.[17,18] Postbiotics derived from *Lactobacillus plantarum* have shown promising antifibrotic effects in in vitro and animal models of liver fibrosis.[19,20] Despite this, research on the effects of postbiotics on OSF remains limited.

Given the comparable pathogenic mechanisms of fibrotic diseases, this study aimed to examine the effect of PostBio GK4, a postbiotic derived from *Lactobacillus pentosus GK4*, on OSF and its underlying mechanism. We hypothesized that PostBio GK4 can inhibit myofibroblast activation induced by arecoline in BMFs by modulating TGF-β and Smad signaling. Elucidating the antifibrotic effects and mechanisms of PostBio GK4 could aid in developing novel therapeutics to mitigate OSF.

Materials and methods

Source and development of strains employed in experiments

The *Lactobacillus pentosus* strain GK4 was provided by Grape KingBio Co. Ltd. (Taoyuan, Taiwan). This strain had been identified using 16S rRNA and extracted from pickled cabbage in Taiwan, and it was subsequently submitted to the Taiwan Bioresource Conservation and Research Center (Bioresource, Hsinchu, Taiwan) with the accession number BCRC 910858. Upon activation, the GK4 strain was cultured in MRS broth (Merck, Darmstadt, Germany) and incubated for 16 h at a temperature of 37 °C to generate a novel liquid solution.[21]

Creation of the PostBio GK4 postbiotic strain

The aseptic fermentation byproducts of GK4, referred to as PostBio GK4, were obtained by centrifuging the newly activated bacterial solution at 5000 revolutions per minute and 25 °C for 10 min using the Centrifuge 5804 R manufactured by Eppendorf AG (Hamburg, Germany). The supernatant was then adjusted to pH 6.8–7.2 and sterilized though 0.22 μl filtration.[22] To ensure its preservation for future use, it should be stored at −20 °C.

OSF tissues and cell culture

The OSF and normal tissue samples were acquired under the consent of patients recruited from the Oral Medicine Center at Chung Shan Medical University Hospital in

Taichung, Taiwan. The research protocol was approved by the Institutional Review Board of Chung Shan Medical University Hospital (IRB: CSMUH No: CS2-21189). Fibroblasts were derived from healthy buccal mucosa (BMFs) while fibrotic BMFs (fBMFs) were obtained from patients with oral submucous fibrosis (OSF). These cells were then cultured following the established criteria and methodology described in a previous publication.[3] Two individuals were selected from donors for this study.

For this study, cell cultures from the third and eight passages were utilized.

Cell viability assay

The cytotoxic effect of PostBio GK4 on fBMFs and BMFs was evaluated by culturing 10,000 cells per well in 24-well plates with varying concentrations of PostBio GK4 or a control substance at a temperature of 37 °C for 48 h. Subsequently, cell viability was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) testing. The blue formazan crystals generated from viable cells were dissolved in DMSO and their absorbance was measured using spectrophotometry at a wavelength of 570 nm. The data were expressed as a percentage relative to the DMSO control for the group treated with 100%. The IC50 values were determined using GraFit software (Erithacus Software Ltd., West Sussex, UK).

Collagen gel contraction assay

Collagen contraction assay (MilliporeSigma, St. Louis, MO, USA) was utilized to measure activated fibroblast collagen contractility. 2×10^5 cells with or without PostBio GK4 for 48 h were suspended in cold collagen solution (2 mg/ml) and dispensed into 24-well plates at 0.5 ml/well, followed by the addition of 1 ml culture media after collagen polymerization. Collagen gels were then gently separated from culture dish sides with a sterile spatula to start contraction. The collagen gel area, representing the contraction index, was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Migration and invasion assays

To assess cell migration and invasion, we utilized a Transwell® system featuring a polycarbonate filter membrane (Corning Inc., Corning, NY, USA) with 8 μm pores, housed within a 24-well plate. For the invasion assay, the membrane was coated with Matrigel. In the upper compartment, 1×10^5 cells were introduced in 250 μL of serum-free medium, while the lower chamber contained 10% fetal bovine serum to act as a chemoattractant. Following a 24-h incubation period, the filter membrane was stained by 0.1% crystal violet solution. Cells were then enumerated from five 100-fold regions using an inverted microscope.

Wound healing assay

Cells were inoculated into 6-well culture dishes, and to induce wound formation, sterile 200 μL plastic pipette tips were used to create incisions, removing the continuous cell layer. After 48 h, cell migration towards the center of the incision was observed using a microscope.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA kit for TGF-β was obtained from R&D Systems (Minneapolis, MN, USA). Arecoline-induced cells were cultured on 6-well plates, treated with different doses of PostBio GK4, and then incubated for 48 h. Cell debris was removed from the cell supernatants through centrifugation. The supernatants were analyzed using ELISA following the manufacturer's instructions.

Western blot analysis

Western blot analysis was conducted as previously described.[23] Antibodies targeting COL1A1, α-SMA, phosph-Smad2, and Smad2 were procured from Santa Cruz Biotechnology, Inc., located in Santa Cruz, CA, USA. Following primary antibody application, membranes underwent secondary antibody treatment. A LAS-1000 plus Luminescent Image Analyzer, manufactured by GE Healthcare (Chicago, IL, USA), was utilized to capture immunoreactive bands generated using an ECL-plus chemiluminescence substrate provided by Perkin-Elmer (Waltham, MA, USA).

Statistical analysis

The statistical analysis was performed using SPSS Statistics version 13.0 (SPSS Inc., Chicago, IL, USA). The statistical significance among the experimental groups was assessed using one-way analysis of variance (ANOVA). Statistical significance was considered as *P*-values below 0.05.

Result

Firstly, we revealed that fibrotic BMFs (fBMFs) exhibited a greater sensitivity to PostBio GK4, as evidenced by a lower IC50 value, Y range and slope factor compared to BMF cells (Fig. 1). IC50 of PostBio GK4 in fBMFs is 22.1% while BMF cells showed significantly higher value, 35.4%. Arecoline is the main component of Areca nuts in inducing OSF environment through the induction of myofibroblast differentiation of BMFs. Thus, in the following experiments, BMFs were exposed to arecoline, and PostBio GK4 of various doses was simultaneously added to examine its therapeutic effects. Considering activated myofibroblasts possess increased contractile, migratory and wound healing ability, we assessed the impacts of arecoline and PostBio GK4 on these parameters. Stimulation of fibroblasts with arecoline significantly induce contraction in cells shown by the reduced gel area (Fig. 2). In addition, arecoline also resulted in increased migration (Fig. 3) and wound healing abilities (Fig. 4). When these arecoline-stimulated BMFs were treated with PostBio GK4, the aforementioned phenomena were reversed markedly. Although the higher dosage of PostBio GK4 (8%) showed slightly more superior effects than the lower dosage (4%), the differences were not significant. Besides that, the protein expression of myofibroblast markers were also examined, it was observed

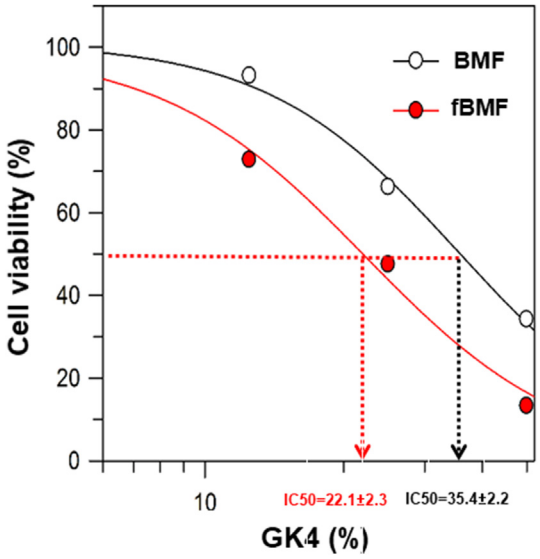

Figure 1 Dose-response curves in buccal mucosal fibroblasts and fibrotic BMFs when treated with PostBio GK4.
Fibrotic BMFs group exhibited higher sensitivity to PostBio GK4, as evidenced by the lower IC50 value, Y range and slope factor as compared to BMFs.

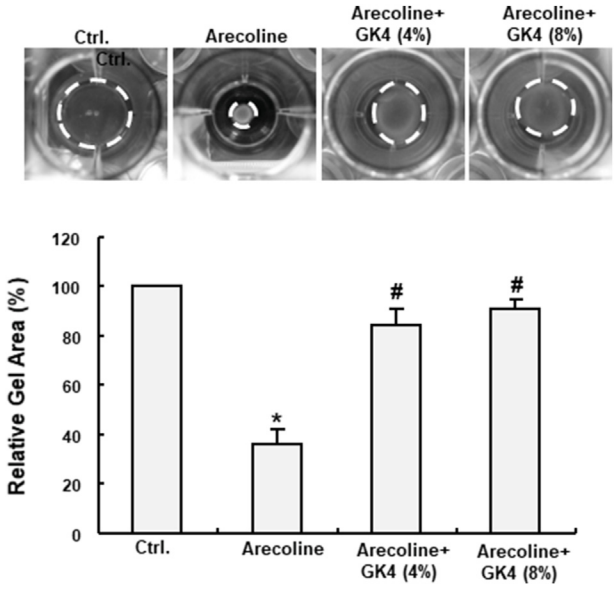

Figure 2 Effect of PostBio GK4 treatments on gel area in arecoline-induced buccal mucosal fibroblasts.
Green dotted lines indicated the area of the gel contraction. Arecoline significantly reduced the gel area while PostBio GK4 at 4% and 8% doses reversed the contractions. Data represent the mean ± SD * *P*-values <0.05 compared to control group; # *P*-values <0.05 compared to Arecoline group.

that COL1A1 and α-SMA were increased in arecoline-induced cells but reversed by the addition of PostBio GK4 in a dose-dependent manner (Fig. 5). Lastly, we investigated if the inhibitory effects of PostBio GK4 on arecoline-induced BMFs is due to the modulation of TGF-β and smad2/3 signaling. As shown in Fig. 6A and B, both the TGF-β

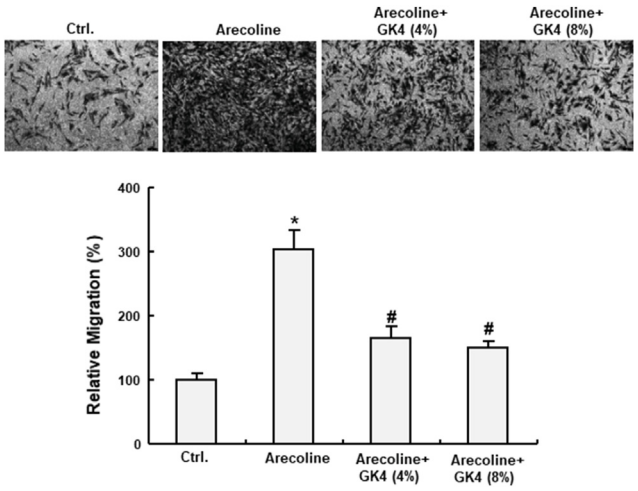

Figure 3 Impact of PostBio GK4 on the migratory ability of arecoline-induced buccal mucosal fibroblasts.
Relative migration was calculated using the proportion of migration of the test groups to the control. The stimulation of arecoline led to a notable rise in the relative migration of the fibroblast, however, the administration of PostBio GK4 significantly reversed the induced effects. Data represent the mean ± SD * *P*-values <0.05 compared to control group; # *P*-values <0.05 compared to Arecoline group.

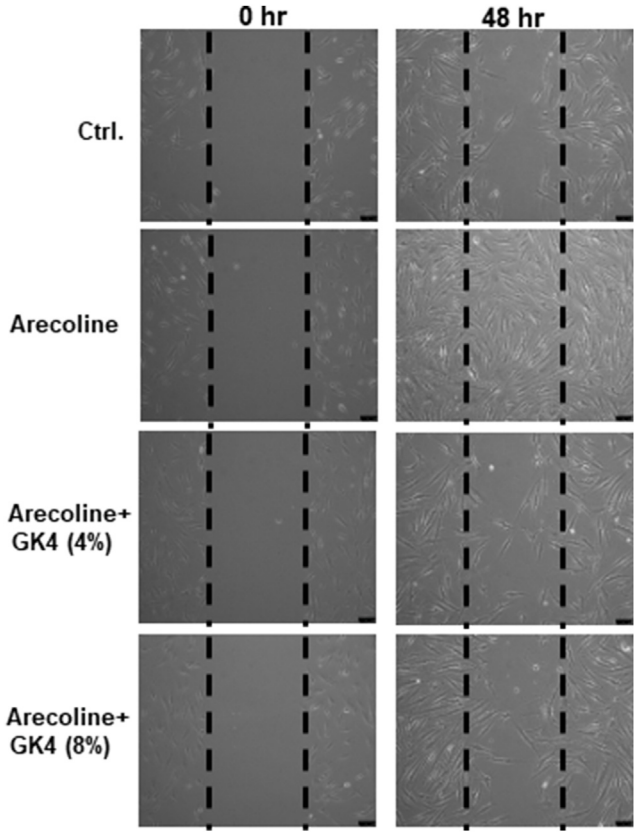

Figure 4 Wound healing assay on buccal mucosal fibroblasts stimulated with arecoline and PostBio GK4.
Wound healing in arecoline-induced BMFs was shown remarkably efficient while treatment with PostBio GK4 at both doses significantly repressed them.

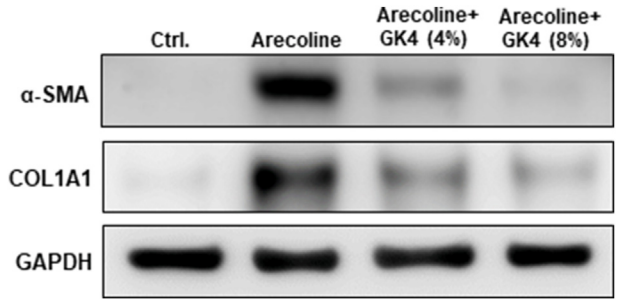

Figure 5 Impact of PostBio GK4 on myofibroblast markers in arecoline-induced buccal mucosal fibroblasts.
The protein levels of α-SMA and COL1A1 were shown the highest when BMFs were cultured with arecoline. The intervention with PostBio GK4 for 48 h treatment inhibited the heightened expression in a dose dependent manner.

secretion and Smad2/3 phosphorylation were found upregulated in arecoline-induced BMFs but were repressed dose-dependently when PostBio GK4 was given.

Discussion

In this study, we explored the role of myofibroblast in precancerous OSF and the therapeutic potential of PostBio GK4 against them in vitro. We observed that PostBio GK4 exhibited greater potency in fibrotic BMFs compared to normal BMFs, suggesting its potential for treating OSF with minimal side effects on normal counterparts. As mentioned earlier, the differentiation of BMFs into myofibroblasts is a key factor in OSF progression. While myofibroblasts play a role in wound healing, their persistent presence in tissues accelerates fibrosis.[7] Myofibroblasts possess enhanced contractile and migratory abilities compared to BMFs.[9,10] Consistent with previous findings,[8] arecoline was found to increase cell contractility, migration and wound healing in this study, whereas treatment with PostBio GK4 reversed these effects. Myofibroblasts exhibit strong contraction and migration due to the presence of cytoskeletal proteins similar to smooth muscle cells, specifically α-smooth muscle actin.[10] As predicted, this study revealed that arecoline-induced BMFs exhibited elevated expression of myofibroblast markers, including α-SMA and COL1A1. Remarkably, treatment with PostBio GK4 suppressed the expression of all these myofibroblast markers. Currently, there is relatively little study on the effects of PostBio GK4 also known as *Lactobacillus pentosus* postbiotics; nevertheless, there are a few works highlighting the immunomodulatory capabilities of *Lactobacillus pentosus* probiotics. For instance, this probiotic has been shown to suppress the development of hepatic inflammation and fibrosis in steatohepatitis murine model,[24] ameliorate gastric inflammation in murine model and modulate high-fat diet induced metabolic disorder in mouse model.[25,26]

Since PostBio GK4 was able to mitigate the myofibroblast activation among fibroblasts induced by arecoline, it is crucial to further elucidate its underlying mechanism. We targeted TGF-β and Smad2/3 signaling pathways in our investigation because these pathways have been observed to mediate the activation of myofibroblast in OSF.[27] In addition, consumption of certain postbiotics has been observed to shield against profibrotic TGF-β and Smad2/3 pathways and inhibition of these pathways effectively repressed arecoline-induced myofibroblast activation.[28] The present study showed PostBio GK4 intervention significantly mitigate the increased expression of TGF-β and phosphorylation of Smad2/3 in a dose dependent manner. TGF-β have been shown to trigger Smad-dependent signaling pathways, boosting the production of extracellular matrix proteins,[29,30] and promoting the transition of fibroblasts into myofibroblasts.[31] Till present, this is the first study to look into the impacts of *Lactobacillus pentosus* postbiotics on OSF and the underlying mechanism, which the findings might serve as a novel platform in designing therapeutics for other similar fibrotic diseases. Nevertheless, further in-vivo studies will be required to fully comprehend PostBio GK4's involvement in modulating these signaling pathway.

In conclusion, our results revealed that PostBio GK4 has a beneficial impact on the arecoline-induced BMFs through the suppression of contractility, migration and wound healing in myofibroblasts. It was proposed that PostBio GK4 wields its anti-fibrotic effect against OSF via the modulation of TGF-β and Smad2/3 signaling pathways. Hence, PostBio GK4 has the potential to mitigate the transition of

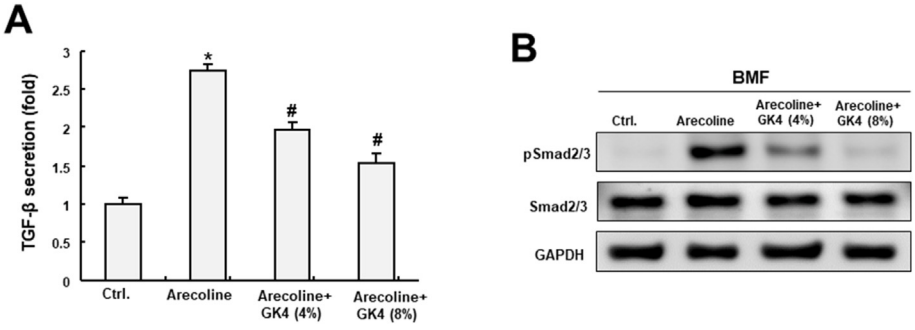

Figure 6 Investigation of TGF-β and Smad2/3 signaling in buccal mucosal fibroblasts exposed to arecoline and PostBio GK4.
(A) The arecoline considerably enhances the secretion levels of TGF-β, while PostBio GK4 resulted in the downregulation of TGF-β secretion dose. (B) The arecoline significantly upregulated the phosphorylation of Smad2/3, while PostBio GK4 reversed the phenomena dose dependently. Data represent the mean ± SD * *P*-values <0.05 compared to control group; # *P*-values <0.05 compared to Arecoline group.

BMFs into myofibroblasts and may be used as an adjunct therapeutic strategy for intervening in OSF.

Declaration of competing interest

The authors have no conflicts of interest to this article.

Acknowledgments

This work was funded by grants from the Chung Shan Medical University Hospital, Taichung, Taiwan (grant number: CSH-2022-C-005); Chung Shan Medical University and Changhua Christian Hospital (grant number: CSMU-CCH-112-09) in Taiwan.

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