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Original Article

Honokiol inhibits arecoline-induced oral fibrogenesis through transforming growth factor-β/Smad2/3 signaling inhibition

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KEYWORDS
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Background/purpose: The habit of areca nut chewing has been regarded as an etiological factor of precancerous oral submucous fibrosis (OSF). In the present study, we aimed to evaluate the anti-fibrosis effect of honokiol, a polyphenolic component derived from *Magnolia officinalis*.
Methods: The cytotoxicity of honokiol was tested using normal and fibrotic buccal mucosal fibroblasts (fBMFs) derived from OSF tissues. Collagen gel contraction, Transwell migration, invasion, and wound healing capacities were examined. Besides, the expression of TGF-β/Smad2 signaling as well as α–SMA and type I collagen were measured as well.
Results: Honokiol exerted higher cytotoxicity of fBMFs compared to normal cells. The arecoline-induced myofibroblast activities, including collagen gel contractility, cell motility and wound healing capacities were all suppressed by honokiol treatment. In addition, the

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expression of the TGF-β/Smad2 pathway was downregulated along with a lower expression of α-SMA and type I collagen in honokiol-receiving cells.
Conclusion: Our data suggest that honokiol may be a promising compound to alleviate the progression of oral fibrogenesis and prevent the transformation of OSF oral epithelium into cancer.
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Introduction

As one of the oral potentially malignant disorders, oral submucous fibrosis (OSF) is characterized by chronic inflammation and deposition of extracellular matrix (ECM), such as collagen, in the oral mucosa. Patients with OSF often suffer from dry mouth, burning sensation, and stiffness of the oral cavity, which results in difficulty of mouth opening and impairment of oral health maintenance. The habit of areca nut chewing has been revealed to play an important role in the pathogenesis of OSF. Also, the components of areca nuts have been demonstrated to induce several inflammatory molecules, such as TGF-β.[1,2] Diverse cellular players orchestrate fibrogenesis after wounding, and myofibroblasts are the primary ECM-secreting cells and responsible for the wound closure.[3] It has been known that the transition of fibroblasts towards the myofibroblastic phenotype, with the acquisition of specialized contractile features, is critical for tissue remodeling. Several studies have demonstrated that the expression of α-smooth muscle actin (α-SMA) in stress fibers enhances the contractile activity of cultured fibroblasts.[4,5] In OSF, the upregulated expression of α-SMA in buccal mucosa obtained from patients with a habit of areca nut chewing is reported.[6] Hence, various therapeutic approaches of OSF have been developed to target myofibroblasts.

Honokiol (3,5′-diallyl-4,2′-dihydroxybiphenyl) and Magnolol (5, 5′-diallyl-2, 2′-dihydroxybiphenyl) are the main substances from the Magnolia bark extract. They are positional isomers with biphenolic groups that have been found to have various pharmacological effects, such as anti-inflammatory,[7] anti-cancer,[8] or anti-fibrosis[9] properties. Previously, we have examined the inhibitory activities of magnolol on inflammation[10] and oral cancer stemness.[11] Besides, we demonstrated that honokiol exerted the chemosensitizing effect to suppress the cancer stemness features in oral cancer.[12] Nevertheless, the treatment efficiency of honokiol in OSF has not been investigated. Given that various reports have shown that application of honokiol was able to inhibit myofibroblast activation,[13,14] we sought to examine if honokiol can impede the areca nut-induced myofibroblasts transdifferentiation.

In the current study, we tested the IC_{50} values of honokiol in normal and fibrotic oral cells and measured various myofibroblast activities as well as the TGF-β signaling after areca nut stimulation. Lastly, we examined the expression of myofibroblast marker, α-SMA, and ECM component, type I collagen, to ascertain the impacts of honokiol on delay the progression of areca nut-associated OSF.

Materials and methods

Reagents and cell culture

Arecoline and collagen solution from the bovine skin were purchased from company (Sigma–Aldrich, St. Louis, MO, USA). Primary cultures including buccal mucosal fibroblasts (BMFs) and patient-derived fibrotic BMFs (fBMFs) were cultivated as previously described and the third and eighth passages were used in this study.[6]

Cell proliferation assay

Cells were plated in wells of 96-well-plate as 1×10^4 cells/well in 0.1% DMSO or different concentrations of honokiol-containing medium and cultured at 37 °C for 48 h. Cell proliferation/survival was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The 570 nm absorbance of the DMSO treated group was set as 100% and data were presented as the percentage of DMSO control.[10]

Collagen gel contraction assay

Cells were suspended in collagen gel solution (Sigma–Aldrich) and added into a 24-well-plate followed by incubation at 37 °C for 2 h. After polymerization, the gels were further incubated within 0.5 ml medium for 48 h. The collagen gel size change (contraction index) was quantified using ImageJ software (NIH, Bethesda, MD, USA).[15]

Cell migration and invasion assays

Cell migration and cell in invasion assays were performed as previously described.[15]

Wound healing assay

Cells were seeded into a 12-well culture dish to reach ~80% confluence. Then the monolayer was scratched with a sterile 200 μL pipette tip across the center of the well to create a denuded area. Cells were allowed to grow for an additional 48 h and stained with crystal violet. Cell movement towards the wound area were photographed at 0 and 24 h under a microscope.[15]

Western blot assay

The extraction of proteins from cells and Western blot analysis were performed as described.[15] Briefly, samples were boiled at 95 °C for 5 min and separated by 10% SDS-PAGE. The proteins were wet-transferred to Hybond-ECL nitrocellulose paper (Amersham, Arlington Heights, IL, USA). The following primary antibodies were used: anti—human α-SMA, COL1A1, smad2, p-smad2 (Cell Signaling, Beverly, MA, USA) and anti—GAPDH (Millipore, Billerica, MA, USA). Immunoreactive protein bands were detected by the ECL detection system (Amersham Biosciences Co., Piscataway, NJ, USA).

Statistical analysis

Statistical Package of Social Sciences software (version 13.0) (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The presented results were representative of three independent experiments with similar results. Statistical differences were evaluated using the Student's *t*-test, and were considered significant at $p < 0.05$.

Results

First, we investigated the effect of honokiol on cell toxicity using normal BMFs and patient-derived fBMFs after 48-h treatment with various concentrations of honokiol using MTT assay. Our results suggested that the IC_{50} values of BMFs and fBMFs were 111.2 ± 13.6 μM and 65.7 ± 9.2 μM (Fig. 1), respectively. This result indicated that a lower concentration of honokiol was sufficient to reduce the cell viability of fBMFs without damaging normal cells. According to Fig. 1, we chose 5, 10, and 20 μM of honokiol (under IC_{50}) for further examination.

Arecoline is a major alkaloid of areca nut that has been proven to induce myofibroblast transdifferentiation of BMFs.[6] Therefore, we treated these arecoline-stimulated BMFs with various concentrations of honokiol to reveal whether honokiol possessed anti-fibrotic property. As shown in Fig. 2A, the arecoline-induced collagen gel contractility of BMFs was dose-dependently reduced in response to honokiol treatment. Moreover, we found that the arecoline-elicited cell migration and invasion were prevented by honokiol treatment in a dose-dependent manner (Figs. 2B and 3A). Similarly, we demonstrated that honokiol was able to impede the wound healing ability of the arecoline-stimulated BMFs in a dose-dependent fashion (Fig. 3B). Altogether, our results suggested that at least 10 μM of honokiol was sufficient to mitigate the myofibroblast activities.

Given that areca nut has been known to enhance the TGF-β/Smad2 signaling and contribute to the pathogenesis of OSF,[1] we sought to evaluate if honokiol could prevent the activation of the TGF-β pathway. As expected, the arecoline-induced TGF-β secretion was suppressed by the increasing concentration of honokiol in BMFs (Fig. 4A). Furthermore, the phosphorylation of Smad2 after arecoline stimulation was inhibited (Fig. 4B), and the type I collagen and α-SMA were downregulated in honokiol-treated cells (Fig. 5). Collectively, we showed that honokiol exerted an anti-fibrotic effect by amelioration of arecoline-elicited myofibroblast activities, possibly via suppression of the TGF-β pathway.

Discussion

In the past few years, numerous natural compounds have been shown to hinder oral fibrogenesis, such as glabridin,[15] epigallocatechin-3-gallate,[16] and arctigenin.[17] In the present study, we showed that honokiol can mitigate several myofibroblast activities, including collagen gel contractility, migration, invasion, and wound healing capacities. It has been demonstrated that honokiol exerts the anti-fibrosis properties via numerous mechanisms. For instance, honokiol has been shown to attenuate tubulointerstitial fibrosis by inhibiting ECM and pro-inflammatory factors.[18] It also has been proven to

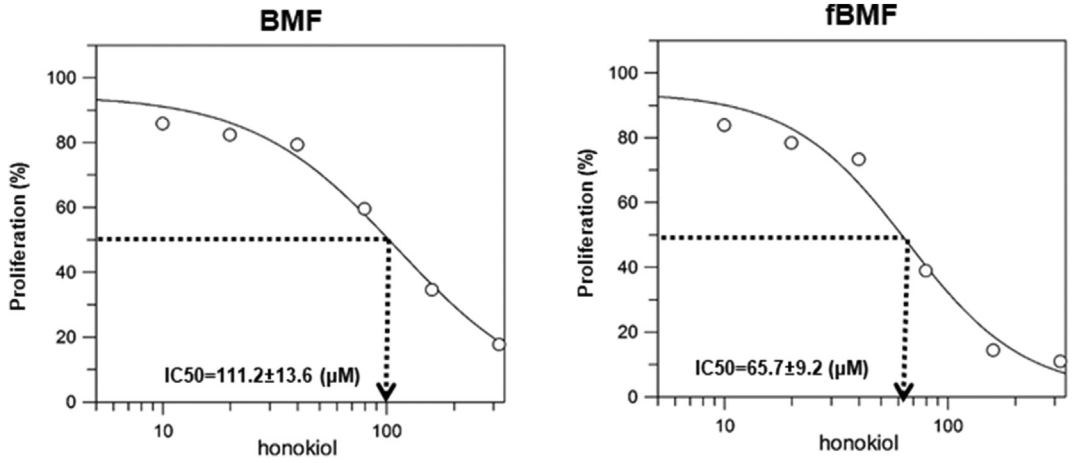

Figure 1 The cytotoxic effect of honokiol on the viability of buccal mucosal fibroblasts (BMFs) and fibrotic BMFs (fBMFs). MTT assay was utilized to examine cell survival/proliferation in response to honokiol. Normal BMFs and fBMFs were seeded at 1×10^4 cells/well and treated with the indicated concentration of honokiol for 2 days (five replicates for each concentration). The IC_{50} values were calculated by GraFit software.

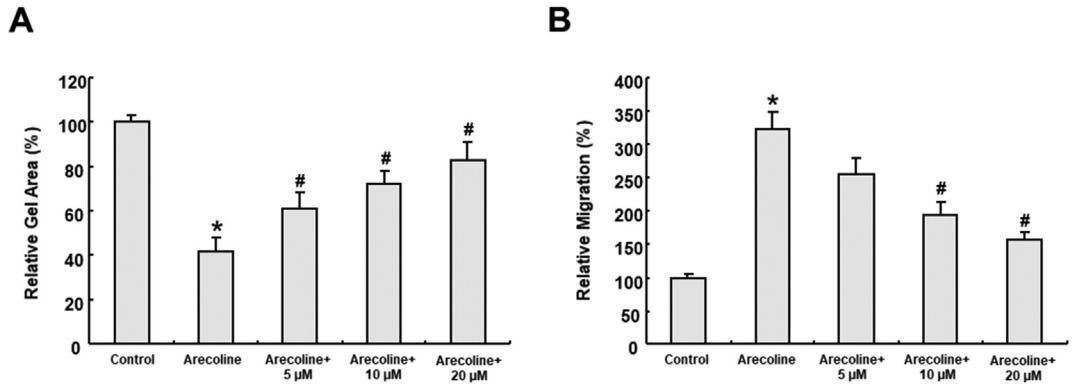

Figure 2 Effects of honokiol on collagen gel contractility and migration capacity in arecoline-stimulated BMFs. The arecoline-treated BMFs were subjected to collagen gel contraction (A) and transwell migration (B) assays. Collagen gel contraction and migration were measured 48 h post-honokiol exposure. The experiments were repeated three times and representative results were shown. Results are means ± SD. *$p < 0.05$ compared to no treatment control group. #$p < 0.05$ compared to the arecoline-only group.

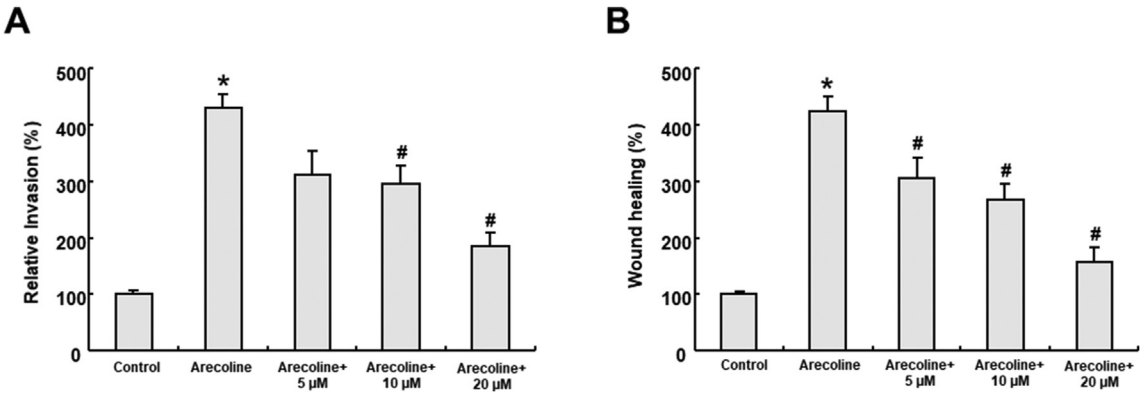

Figure 3 Effects of honokiol on invasion and wound healing abilities (A) Transwell matrix invasion assay was conducted after various concentrations of honokiol were added into the arecoline-treated BMFs. The experiments were repeated three times and representative results were shown. Results are means ± SD. *$p < 0.05$ compared to no treatment group. #$p < 0.05$ compared to the arecoline-only group.

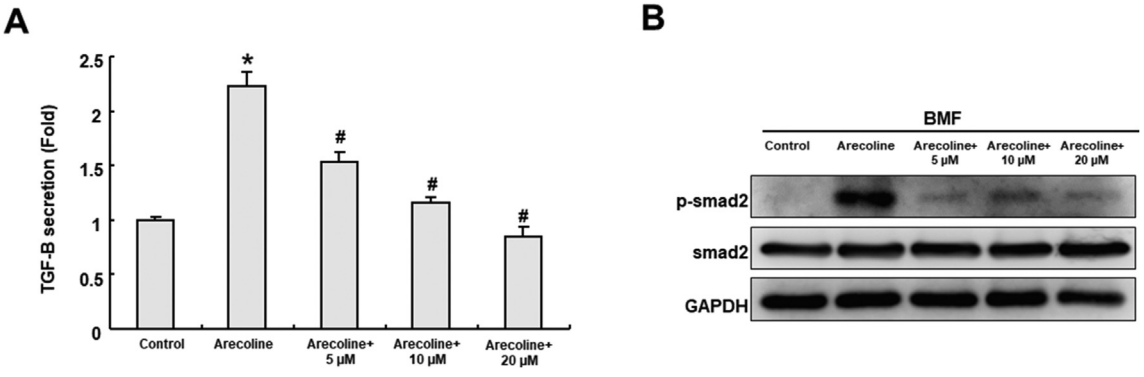

Figure 4 Effects of honokiol on the TGF-β/Smad2 signaling (A) ElISA assay was applied to analyze the TGF-β secretion in response to arecoline treatment with or without honokiol was examined (B) The expression of total Smad2 and phosphorylated Smad2 were assessed in the arecoline-stimulated BMFs along with various concentrations of honokiol. The experiments were repeated three times and representative results were shown. Results are means ± SD. *$p < 0.05$ compared to no treatment group. #$p < 0.05$ compared to the arecoline only group.

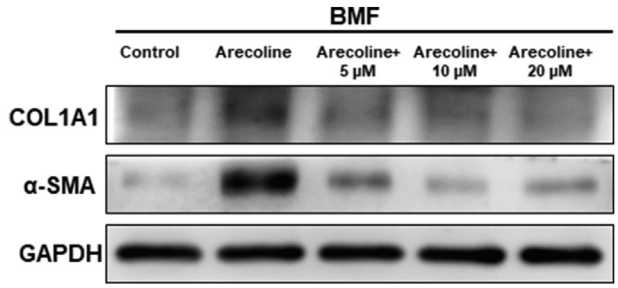

Figure 5 Honokiol treatment downregulates the expression of fibrosis markers. Western blot analysis was used to analyze the protein expression of type I collagen and α-SMA in the arecline-stimulated BMFs with various concentrations of honokiol. The experiments were repeated three times and representative results were shown. Results are means ± SD. $^{*}p < 0.05$ compared to no treatment group. $\#p < 0.05$ compared to the arecoline-only group.

ameliorate liver fibrosis through activation of GSK3β and repression of Wnt3a/β-catenin signaling.[9] Besides, honokiol has been found to block the differentiation of cardiac fibroblasts via an increase of Sirt3 activity.[13] Another study showed that honokiol downregulates the expression levels of type I collagen, type III collagen, and α-SMA in hypertrophic scar-derived fibroblasts through TGF-β/Smad2 signaling.[14] Similarly, it has been shown that honokiol suppressed the TGF-β1-induced proliferation of fibroblasts derived from the epidural scar tissues as well as the expression of ECM components and connective tissue growth factor by inhibiting the phosphorylation and nuclear translocation of Smad2/3.[19] In line with these studies, we demonstrated that honokiol alleviated the myofibroblast features via suppression of TGF-β/Smad2 signaling. We showed that the production of TGF-β was enhanced by arecoline stimulation, which was inhibited by honokiol treatment. These findings suggested that honokiol possesses the ability to impede the TGF-β/Smad pathway via both downregulations of TGF-β secretion and nuclear translocation of Smads.

Aside from the anti-fibrosis effect, honokiol has been utilized to ameliorate the aggressiveness of oral cancer. It has been shown that honokiol exhibits higher antiproliferation activities than magnolol,[20] and inhibits tumor growth by induction of apoptosis, cell cycle arrest, and autophagy using oral cancer cell lines.[8] Besides, honokiol has been shown to reduce the *in vitro* sphere formation capacity and *in vivo* tumor growth of oral cancer stem cells (OCSCs) via inhibition of JAK/Stat signaling.[21] Our previous work was consistent with these results and showed honokiol sensitized the chemotherapy via regulation of IL-6/Stat3 in OCSCs.[12] In fact, IL-6 has been shown to mediate the myofibroblastic differentiation in OSF.[22] Additionally, the TGF-β1/IL-6 pathway has been shown to affect the progression of oral cancer.[23] As such, further studies are required to elucidate the mechanisms underlying the regulation between TGF-β1/IL-6 signaling and myofibroblast activation.

In summary, our results revealed that honokiol may serve as a promising candidate to inhibit the progression of OSF into oral cancer without damaging normal fibroblasts. Subsequent *in vivo* studies are necessary to translate these findings into clinical application.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgment

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