Journal of Dental Sciences 20 (2025) 1129–1138

Available online at www.sciencedirect.com

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Original Article

Hypoxia amplifies arecoline-induced invasion and metastasis in oral squamous cell carcinoma — Insights into TGF-β1 signaling and collagen production

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Received 28 October 2024; Final revision received 25 December 2024
Available online 24 January 2025

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https://doi.org/10.1016/j.jds.2024.12.026

KEYWORDS
Betel quid;
Arecoline;
Hypoxia;
Type I collagen;
TGF-β1;
Oral squamous cell carcinoma

Abstract *Background/Purpose:* Betel quid chewing is a major risk factor for oral squamous cell carcinoma (OSCC), largely due to arecoline, a key alkaloid. Hypoxia, common in the tumor microenvironment, also influences cancer progression. This study investigated the combined effects of arecoline and hypoxia on proliferation, migration, and protein expression in tongue squamous cell carcinoma (SCC-25) cells, focusing on the TGF-β1 signaling pathway and type I collagen production.
Materials and methods: SCC-25 cells were treated with arecoline and incubated for 24 h under normoxia or hypoxia. Cytotoxicity assays and Western blotting were performed to assess cell viability and protein expression.
Results: At 2.5 μg/mL, arecoline enhanced SCC-25 cell proliferation under normoxia, while hypoxia suppressed this effect. Arecoline significantly promoted cell migration that was further amplified by hypoxia. Western blotting revealed that arecoline upregulated TGF-β1, Smad2/3, phosphorylated Smad2/3, and type I collagen. Under hypoxia, HIF1-α expression increased along with TGF-β1 and type I collagen, indicating that hypoxia enhances arecoline-induced collagen production through TGF-β1 signaling.
Conclusion: Arecoline stimulates SCC-25 cell proliferation and migration, with hypoxia amplifying these effects by promoting TGF-β1 signaling and type I collagen production. These findings suggest that betel quid consumption, in combination with hypoxia, may exacerbate the invasion and metastasis of OSCC.

Introduction

Chewing betel quid is a significant risk factor for oral cancer, with the International Agency for Research on Cancer classifying it as a Group 1 carcinogen.[1] Arecoline, a major alkaloid found in the areca nut, has been shown to inhibit p53, repress DNA repair, and trigger the DNA damage response in human epithelial cells. This accumulation of DNA damage contributes to the initiation and progression of oral squamous cell carcinoma (OSCC).[2,3]

In addition to its genotoxic effects, low doses of arecoline promote OSCC cell proliferation by accelerating cell cycle progression and upregulating oncogenes such as c-Myc.[4] This increase in cell proliferation contributes to the rapid expansion of malignant cells in the oral cavity, promoting tumor growth. Arecoline has also been found to induce the transforming growth factor-beta (TGF-β) pathway and epithelial–mesenchymal transition (EMT), processes in which epithelial cells lose their adhesion properties and gain migratory and invasive characteristics.[5,6] Through the activation of EMT markers such as Slug, arecoline enhances the migratory and invasive potential of OSCC cells.[7,8]

Moreover, arecoline disrupts the extracellular matrix (ECM) balance by stimulating the production of collagen and other ECM components via upregulation of the TGF-β1 signaling pathway.[9,10] The excessive deposition of ECM not only leads to fibrosis, as seen in oral submucous fibrosis (OSF), but also creates a rigid tumor microenvironment that supports cancer cell invasion and metastasis.[11] The role of collagen production in tumor progression is particularly important, as it facilitates the structural changes necessary for cancer cell migration and invasion.[12]

Hypoxia, or reduced oxygen levels, is a common feature of the tumor microenvironment and plays a crucial role in cancer progression. Hypoxia is especially linked to metastasis, as it triggers various molecular changes that enable cancer cells to escape the primary tumor and invade distant tissues. The key mediator of the cellular response to hypoxia is hypoxia-inducible factor-1α (HIF-1α), a transcription factor that regulates genes involved in survival, angiogenesis, and metabolism under low oxygen conditions.[13,14] HIF-1α drives tumor progression by promoting angiogenesis through the upregulation of vascular endothelial growth factor (VEGF), facilitating the development of new blood vessels to support tumor growth and metastasis.[15–17]

In addition to its role in angiogenesis, HIF-1α enhances the migratory and invasive capacities of cancer cells by regulating EMT. Studies have demonstrated that HIF-1α interacts with several key pathways, including the TGF-β1 signaling pathway, which is involved in cancer cell migration and ECM remodeling.[16] Under hypoxic conditions, HIF-1α cooperates with TGF-β1 to drive the expression of fibrotic genes such as type I collagen, which stiffens the ECM and further enhances the invasive potential of cancer cells.[15,16]

The interaction between hypoxia and TGF-β1 signaling is especially relevant in OSCC, as both pathways contribute to the aggressive nature of this disease. TGF-β1, in conjunction with HIF-1α, fosters a tumor microenvironment that supports invasion and metastasis, making these pathways key targets for therapeutic intervention. Investigating how hypoxia and arecoline, a major carcinogenic component of betel quid, interact to influence these pathways may provide new insights into the mechanisms driving OSCC progression.[17]

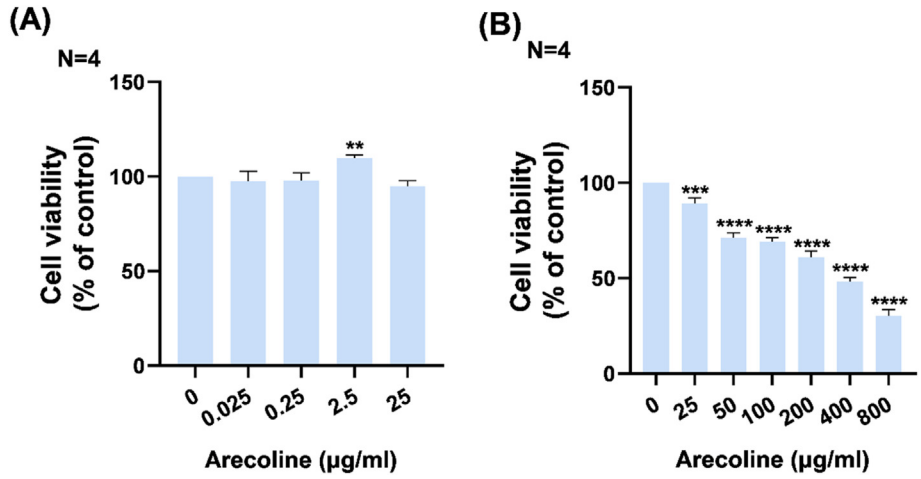

Figure 1 Dose–response of arecoline on SCC-25 cells. The cytotoxic effects of arecoline on SCC-25 cells were measured using the CCK-8 assay. SCC-25 cells were incubated with varying concentrations of arecoline (0, 0.025, 0.25, 2.5, 25, 50, 100, 200, 400, 800 μg/mL) for 24 h. (A) Arecoline at 2.5 μg/mL significantly enhances the proliferation of SCC-25 cells. (B) Arecoline induces a dose-dependent cytotoxic response in SCC-25 cells at higher concentrations. Data are presented as mean ± SE, with experiments performed in triplicate. Statistical significance was determined using a *t*-test. Significant differences are indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Previous studies have focused on HIF-1α expression in OSCC, but no prior research has investigated the combined effects of arecoline and hypoxia on OSCC in vitro under hypoxic conditions. Given that hypoxia upregulates HIF-1α, enhancing tumor cell migration, angiogenesis, and EMT, it is plausible that hypoxic conditions could amplify arecoline's tumor-promoting effects. The potential interaction between arecoline and hypoxia, particularly through the TGF-β1 pathway, has not been thoroughly explored. Understanding this interaction could offer valuable insights into OSCC metastasis.

The objective of this study is to investigate the combined effects of arecoline and hypoxia on proliferation, migration, and TGF-β1 signaling in tongue squamous cell carcinoma (SCC-25) cells. Specifically, this research focuses on how arecoline, in combination with hypoxic conditions, influences type I collagen production, a critical component of the ECM in OSCC progression. By elucidating the interaction between arecoline and hypoxia, this study aims to fill a significant gap in the understanding of OSCC and provide a basis for therapeutic strategies targeting these pathways.

Materials and methods

Cell culture

SCC-25 (tongue squamous cell carcinoma SCC-25 Cells were procured from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in DMEM/F12 with 25 mM HEPES, 400 ng/mL hydrocortisone (Sigma–Aldrich, Merck, Darmstadt, Germany), and 10% FBS.

All cell lines were maintained at 37 °C with 95% air and 5% CO2. Subculturing was performed using 0.05% Trypsin–EDTA (GeneDireX, Taoyuan, Taiwan) when cells reached 80–90% confluence.

SCC cells of **hypoxia** group were incubated in the multi-gas hypoxia incubator (MCO-50 M, PHCbi, Tokyo, Japan) under the condition of 1% O_2, 5% CO_2, and balanced N_2.

Arecoline treatment

Cells were seeded to 80–90% confluence and treated with varying arecoline (Thermo Fisher Scientific, Waltham, MA, USA) concentrations (0–800 μg/mL) for 24 h. Samples were collected for cytotoxicity analysis and to determine appropriate concentrations for subsequent experiments.[2,3]

Cytotoxicity assay

1×10^4 cells/well were seeded in a 96-well plate and treated with arecoline until the cells reached to 80–90% confluence. Cells were then treated with arecoline for 24 h in the normoxia incubator and hypoxia incubator respectively. Following the Cell Counting Kit-8 (Biotools Co., New Taipei City, Taiwan) protocol, the CCK-8 reagent was added, and plates were incubated at 37 °C, 5% CO2 for 1.5 h. Absorbance was measured at 450 nm.

Migration assay

Following the operating instructions for the *Culture-Inserts 2 Well for self-insertion*[18–20] (ibidi, Martinsried, Germany), we first placed the culture insert into a 24-well plate. We then added 70 μL of SCC-25 cells at a density of 6×10^5 cells/mL to each well of the culture insert. Outside the insert, 500 μL of culture medium was added to each well. After allowing the cells to adhere overnight, medium containing arecoline was added for a 24-h treatment. The hypoxia group was incubated in a hypoxia chamber for 24 h following arecoline administration. Photographs were taken at 0 and 24 h, followed by quantitative analysis using ImageJ software.

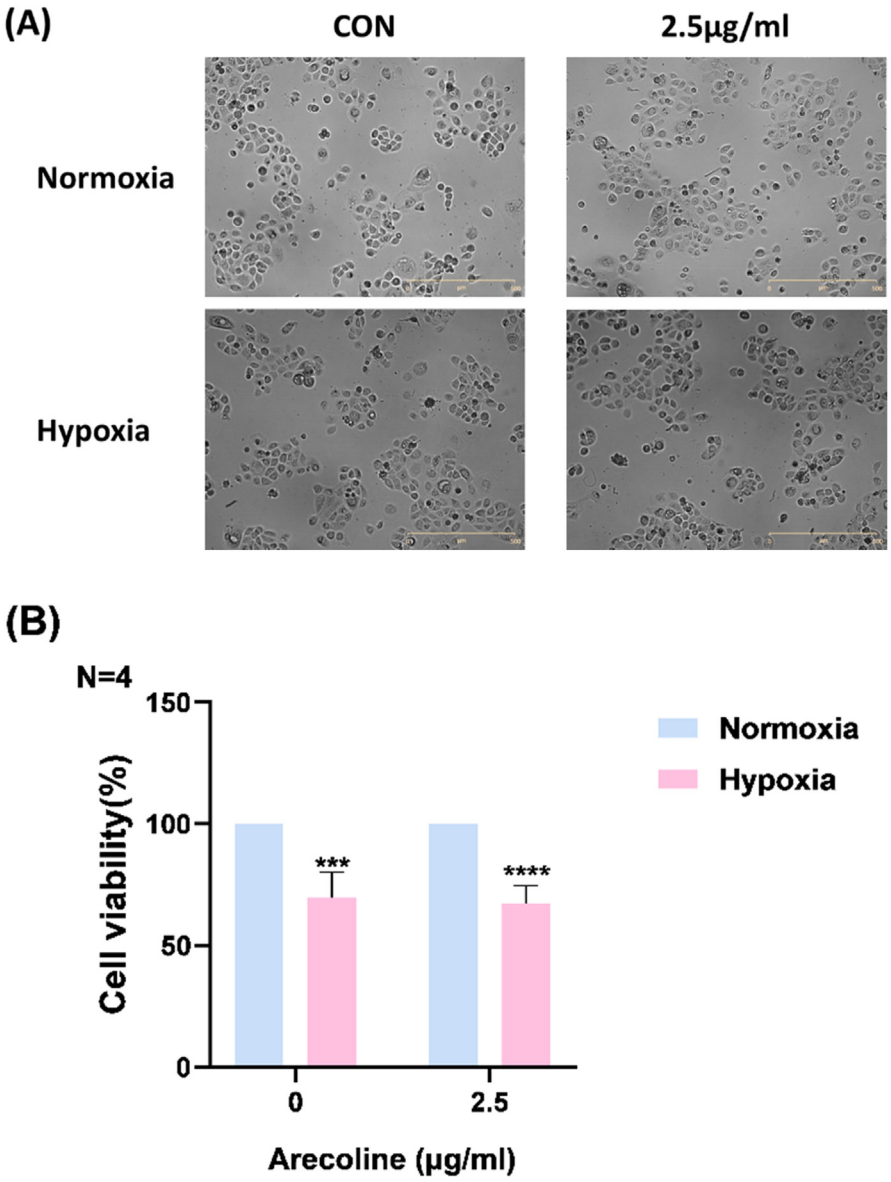

Figure 2 Hypoxia suppresses SCC-25 cell proliferation. (A) SCC-25 cells were treated with 2.5 μg/mL arecoline under hypoxic conditions for 24 h. Images were captured using the JuLI FL cell history recorder at 4X magnification. (B) Hypoxia significantly inhibits the proliferation of SCC-25 cells compared to normoxic conditions, both in the presence and absence of arecoline. Data are presented as mean ± SE, with experiments performed in quadruplicate. Statistical significance was determined using a t-test, with significant differences indicated as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Protein extraction

1×10^6 cells/well were seeded in a 10 cm dish. After 24 h of treatment either under normoxia or hypoxia, cells were washed with 1xPBS, scraped, centrifuged, and lysed on ice with cell lysis buffer, Pro-prep protein extraction solution (iNtRon Biotechnology, Kirkland, WA, USA). The lysate was centrifuged, and the supernatant was collected for protein concentration measurement using the SMART BCA protein assay kit (iNtRon Biotechnology). Samples were stored at −80 °C.

Western blotting

Cell lysate was mixed with 5x protein loading dye (iNtRon Biotechnology), and proteins were separated using 8% SDS-PAGE, then transferred to PVDF membranes (Cytiva, Amersham, UK). Membranes were blocked with 2% BSA (Sigma−Aldrich, Merck, Darmstadt, Germany) and incubated overnight with primary antibodies for TGF-β1 (Abcam, Cambridge, UK), collagen I (Proteintech, Rosemont, IL, USA), HIF1-α (Proteintech), Phospho-Smad2 (Cell Signaling Technology, Danvers, MA, USA), Smad2/3

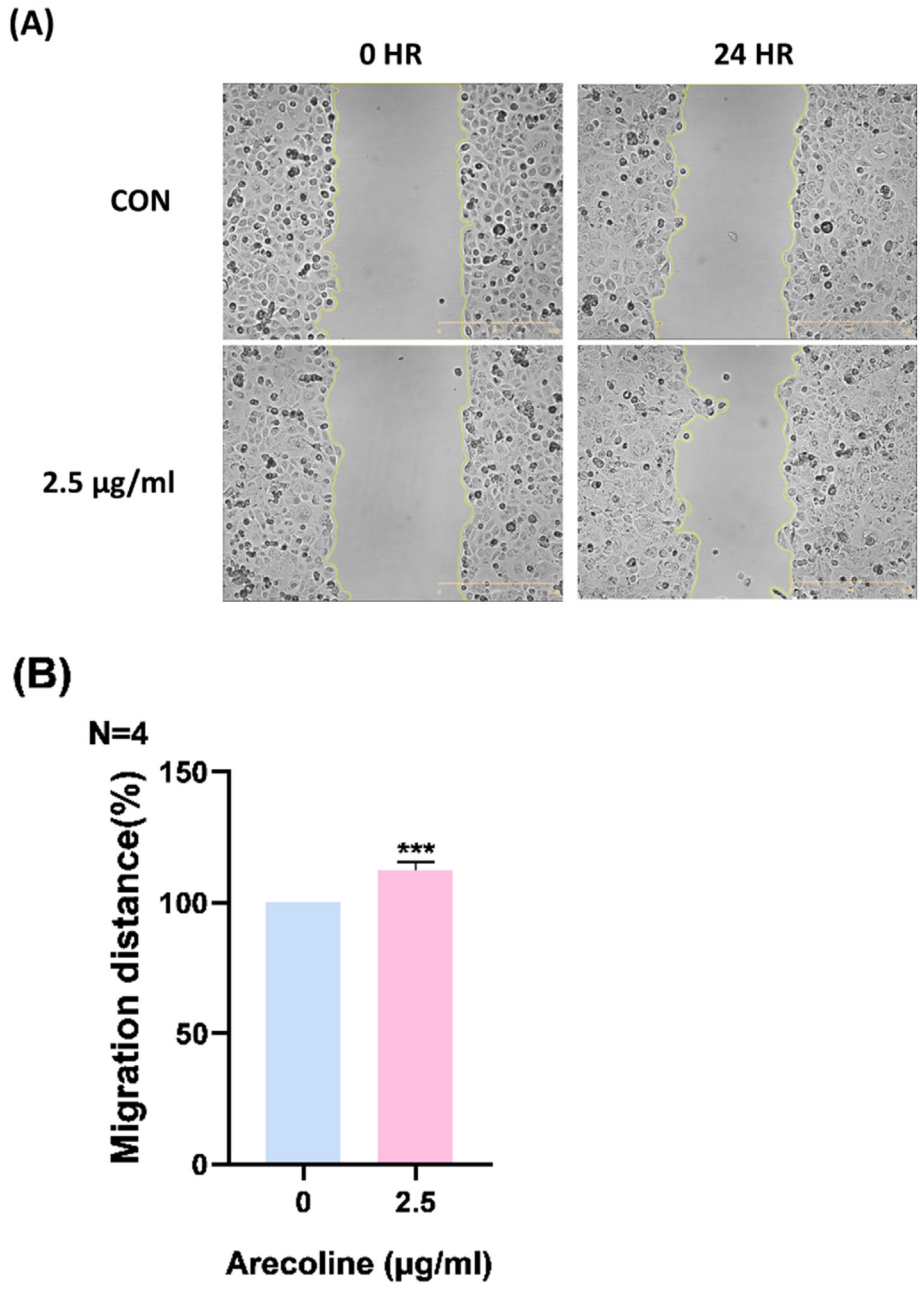

Figure 3 Arecoline Enhances SCC-25 Cell Migration. (A) SCC-25 cells were seeded into Culture-Inserts 2 Well (ibidi, Martinsried, Germany) for a migration assay. After treatment with 2.5 μg/mL arecoline for 24 h, images of cell migration were captured using the JuLI FL cell history recorder at 4X magnification. (B) Arecoline significantly increases the migration distance of SCC-25 cells, promoting cell migration in comparison to the control. Data are presented as mean ± SE, with experiments performed in quadruplicate. Statistical significance was determined using a t-test. Significant differences are indicated as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

(Cell Signaling Technology) and β-actin (Abcam). After washing, membranes were incubated with secondary antibodies (Abcam), and signals were developed using ECL-HRP substrate (Biotools). Images were captured using the Bio-rad ChemiDoc XRS + system (Bio-rad, Hercules, CA, USA) and analyzed with Bio-rad Image Lab Software.

Statistical analysis

Data are expressed as mean ± SE. Statistical analysis was performed using Prism8 software (GraphPad Software, San Diego, CA, USA). Differences between groups were assessed using one-way ANOVA or Unpaired t-tests, with $P < 0.05$ considered statistically significant.

Results

Low concentration of arecoline enhanced proliferation of SCC-25 cells

The cytotoxicity of arecoline on SCC-25 cells was investigated following a 24-h treatment with various concentrations of arecoline. The results showed that 2.5 μg/mL of arecoline enhanced the proliferation of SCC-25 cells (Fig. 1A). Higher concentrations of arecoline exhibited cytotoxic effects on SCC-25 cells in a dose-dependent manner (Fig. 1B). Based on these findings, 2.5 μg/mL of arecoline can stimulate SCC-25 cell proliferation.

Hypoxia inhibited SCC-25 cell proliferation

The effects of 24-h arecoline treatment on SCC-25 cell proliferation under both normoxic and hypoxic conditions were investigated. At a concentration of 2.5 μg/mL, arecoline enhanced SCC-25 cell proliferation under normoxia, whereas proliferation was inhibited under hypoxia (Fig. 2A). The results indicate that hypoxia suppresses SCC-25 cell proliferation compared to normoxia. Furthermore, the administration of arecoline under hypoxic conditions also inhibited SCC-25 cell proliferation (Fig. 2B).

Hypoxia amplified the arecoline-treated SCC-25 cell migration

Cell migration assay was conducted to determine whether arecoline enhances SCC-25 cell migration. The data show that following 24-h arecoline treatment, SCC-25 cells migrated a greater distance compared to the control group, indicating that arecoline induces cell migration (Fig. 3A and B). Additionally, hypoxia alone was shown to enhance SCC-25 cell migration, and when combined with arecoline under hypoxic conditions, the effect was further amplified. This suggests that hypoxia augments the migratory effects of arecoline on SCC-25 cells (Fig. 4A and B). In summary, these findings imply that continued betel quid consumption, combined with prolonged exposure to low oxygen levels, could stimulate cancer cell metastasis in patients with oral squamous cell carcinoma.

Arecoline stimulated type I collagen production under hypoxia: insights into the TGF-β pathway

The protein expression levels of TGF-β1, Smad 2/3, p-Smad2/3, and type I collagen were evaluated using western blotting to assess the effect of arecoline. The data showed upregulation of TGF-β1, Smad 2/3, p-Smad2/3, and type I collagen, suggesting that arecoline stimulates the phosphorylation of Smad2/3 through the upregulation of TGF-β1. This leads to enhanced type I collagen production, thereby influencing the progression of oral cancer (Fig. 5A and B).

Furthermore, the expression of TGF-β1, Smad 2/3, p-Smad2/3, type I collagen, and HIF1-α was also investigated by western blotting to examine the effects of arecoline under hypoxia on type I collagen expression via the TGF-β1 pathway. The results demonstrated that hypoxic conditions stimulate HIF1-α expression, which in turn promotes the protein expression of TGF-β1 and type I collagen. These findings indicate that hypoxia can further stimulate type I collagen production, potentially accelerating the progression of oral cancer (Fig. 5A and C).

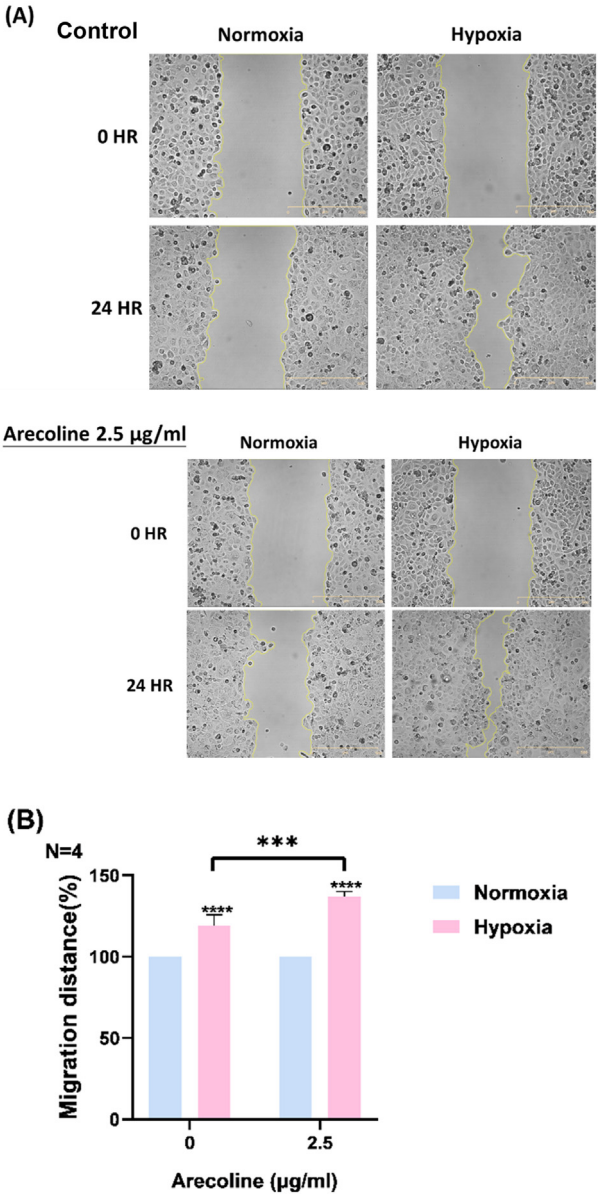

Figure 4 Hypoxia enhances the effect of arecoline on SCC-25 cell migration. (A) SCC-25 cells were treated with 2.5 μg/mL arecoline and incubated under normoxic and hypoxic conditions. Images were captured at 0 and 24 h using the JuLI FL cell history recorder at 4X magnification. (B) Hypoxia significantly enhances the migration of SCC-25 cells treated with arecoline. Data are presented as mean ± SE, with experiments performed in quadruplicate. Statistical significance was determined using a t-test. Significant differences are indicated as follows: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

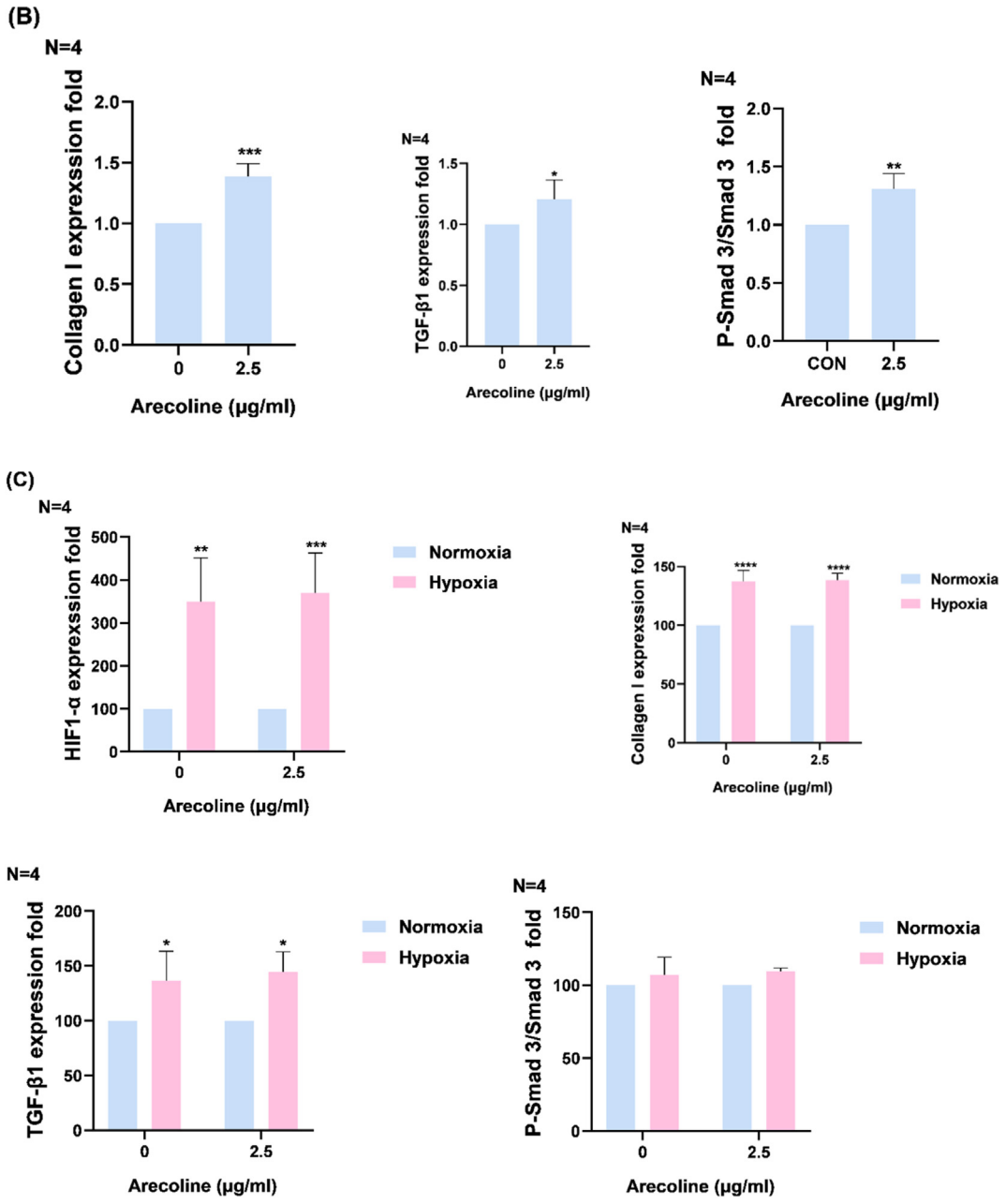

Figure 5 (*continued*).

Discussion

This study demonstrates that arecoline promotes the proliferation and migration of SCC-25 cells, with these effects significantly enhanced under hypoxia. Notably, hypoxia amplified arecoline-induced TGF-β1 and type I collagen expression, suggesting a synergistic interaction between arecoline and hypoxia.

Our results showed that low concentrations of arecoline enhanced the proliferation of SCC-25 cells. This finding aligns with recent studies that indicate low-dose arecoline promotes OSCC cell proliferation by accelerating cell cycle progression, whereas high doses are cytotoxic to OSCC cells.[23] The mechanism by which arecoline promotes OSCC cell proliferation may involve upregulation of c-Myc and downregulation of miR-22, leading to increased oncostatin M expression.[4,21]

Hypoxia-inducible factor-1α (HIF-1α) expression has been shown to be significantly elevated in OSCC specimens compared to normal tissue.[22] Arecoline has also been reported to elevate HIF-1α expression in a dose- and time-dependent manner in areca quid-associated OSCC.[24] In our study, hypoxia inhibited SCC-25 cell proliferation, consistent with Kang et al., who found that hypoxia simulated with deferoxamine mesylate (DFO) significantly reduced the proliferation of hypoxic SCC-15 cells compared to normoxic cells. DFO treatment also resulted in an increased G1 phase population and decreased S/G2 phase population under hypoxia.[25]

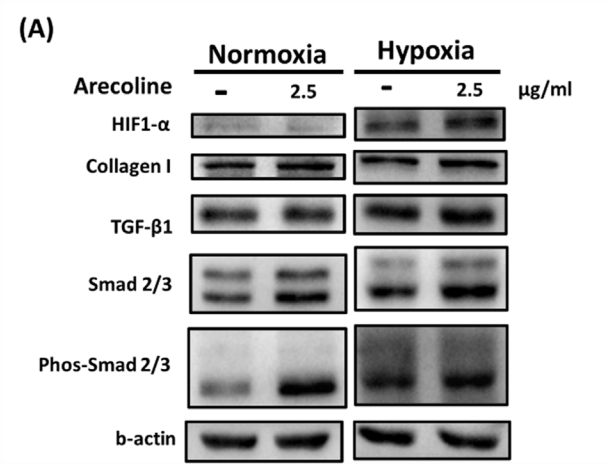

Figure 5 Protein expression in SCC-25 cells treated with arecoline under hypoxia. (A) Western blot analysis of protein expression levels for HIF1-α, type I collagen, TGF-β1, and phosphorylated Smad2/3 in SCC-25 cells treated with 2.5 μg/mL arecoline under normoxic and hypoxic conditions. β-actin was used as the loading control. (B) Arecoline at 2.5 μg/mL upregulates the protein levels of type I collagen, TGF-β1, and phosphorylated Smad3 in SCC-25 cells under normoxia. (C) Hypoxia alone significantly increases the expression of HIF1-α, type I collagen, and TGF-β1 in both control and arecoline-treated SCC-25 cells, with a further enhancement seen in the arecoline-treated group. Data are presented as mean ± SE ($n = 4$). Statistical significance was determined using a t-test, with significant differences indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Our results also demonstrated that arecoline enhances SCC-25 cell migration, consistent with Ren et al., who found that arecoline induces epithelial–mesenchymal transition (EMT) and promotes oral cancer metastasis through serum amyloid A1 (SAA1) expression.[26] Furthermore, recent studies have suggested a strong link between arecoline, cuproptosis, and cancer-associated fibroblasts (CAFs), indicating these factors may play a critical role in OSCC metastasis.[27]

HIF-1α expression is also closely correlated with lymph node metastasis in OSCC.[28] In our study, hypoxia significantly enhanced the migration of SCC-25 cells treated with arecoline. This finding is in line with the study by Kim et al., which showed that hypoxia disrupts tight junctions and promotes OSCC metastasis through the loss of partitioning-defective protein 3 (par3).[29]

Western blot analysis in our study indicates that arecoline stimulates Smad2/3 phosphorylation via the upregulation of TGF-β1 and enhances type I collagen production. High doses of arecoline have also been shown to increase collagen type I accumulation in animal models.[30] Zeng et al. reported that arecoline significantly upregulates α-SMA, COL1A, CTGF, and TGF-β1 expression, promoting Smad3 phosphorylation.[9] Moreover, arecoline was found to upregulate Slug, which binds to the E-box of type I collagen, leading to increased collagen expression.[8] Fang et al. demonstrated that arecoline-induced myofibroblast differentiation occurs via LINC00974-mediated activation of the TGF-β signaling pathway.[31] Similarly, Zhu et al. showed that hypoxic head and neck squamous cell carcinoma (HNSCC) cells promote CAF-like differentiation by secreting TGF-β and small extracellular vesicles (sEVs) containing elevated levels of miR-192/

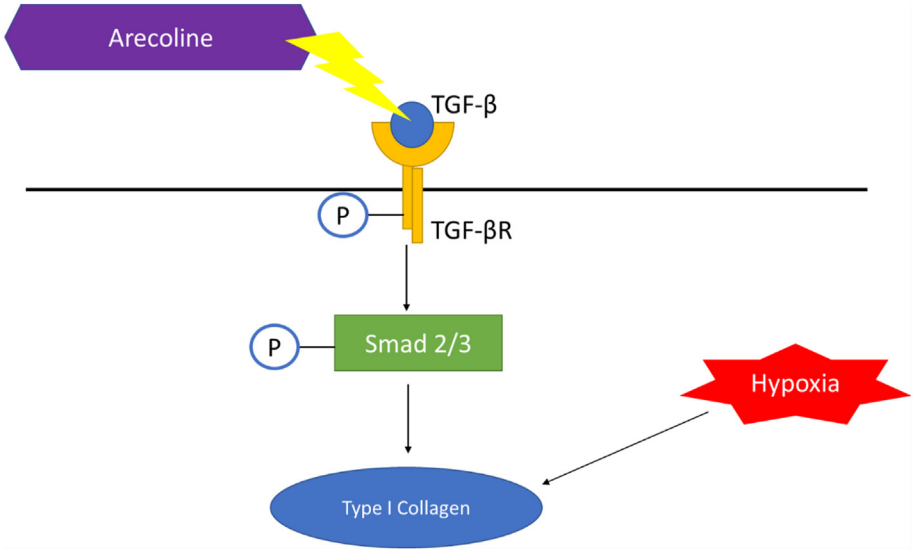

Figure 6 The signaling pathway for collagen type I expression in arecoline-treated SCC-25 cells under hypoxia. Under normoxic conditions, arecoline stimulates the TGF-β1 signaling pathway by activating TGF-β receptors (TGF-βR), leading to phosphorylation of Smad2/3 and downstream production of type I collagen. This process promotes SCC-25 cell proliferation and migration. In a hypoxic environment, the expression of HIF-1α is upregulated, which further stimulates the production of type I collagen. Hypoxia inhibits SCC-25 cell proliferation but enhances migration.

215 family miRNAs.[32] Interestingly, Baumann et al. found that HIF-1α promotes glomerulosclerosis and regulates COL1A2 expression through interactions with Smad3. In their kidney model, TGF-β not only increased HIF-1α levels but also enhanced HIF-1α binding to the COL1A2 promoter and activated the HIF-1α N-terminal transactivation domain,[33] a finding consistent with our results.

However, our study has some limitations, including the use of a single cell line (SCC-25), which may not fully capture the heterogeneity of OSCC across patients or different anatomical sites. Moreover, the concentrations of arecoline used may not correspond directly to the physiological levels found in betel quid chewers. There is no in vivo validation, which limits the applicability of the findings to clinical contexts or physiological levels of arecoline and hypoxia.

We focused heavily on the TGF-β1 pathway without sufficiently exploring other pathways that may be involved in arecoline and hypoxia-mediated effects, such as the PI3K/AKT pathway. The role of other key players, such as CAFs and immune cells in the tumor microenvironment, is not explored, despite the findings suggesting their involvement. Our study does not provide clinical OSCC data or patient samples to support the relevance of the findings in real-world scenarios. The absence of correlation between arecoline levels in betel quid chewers and the experimental concentrations used in the study weakens the translational value of our research.

Future research should focus on investigating the in vivo role of arecoline and hypoxia in OSCC progression to validate these findings. Additionally, exploring the therapeutic potential of targeting the TGF-β1 and HIF-1α pathways in OSCC treatment is warranted. Public health implications should also consider the exposure of betel quid chewers to poorly ventilated or hypoxic environments.

In conclusion, this study highlights the complex interplay between arecoline, hypoxia, and the TGF-β1 signaling pathway in promoting OSCC progression. Hypoxia enhances arecoline-induced cell migration and may modulate the extracellular matrix via HIF-1α, although its direct influence on TGF-β1 signaling requires further investigation (Fig. 6). Future studies should aim to elucidate the molecular mechanisms by which HIF-1α and TGF-β1 interact under hypoxic conditions to drive OSCC invasion and metastasis.

Declaration of competing interest

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

Acknowledgments

This study was financially supported by Kaohsiung Medical University Hospital, Taiwan (KMUH111-1T13). The Liberty Lab, a non-profit research facility, is dedicated to fostering interinstitutional and international collaborations. We are open to and grateful for any grants or support to further advance our research efforts.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2024.12.026.

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