Triphala extract negates arecoline-induced senescence in oral mucosal epithelial cells in vitro

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Abstract

Background: Arecoline found in areca nut causes oral submucous fibrosis. Triphala is an Ayurvedic medicinal preparation used to improve overall physical wellness that has also been shown to improve oral health.

Objectives: To assess the activity of Triphala extract on arecoline-induced senescence in oral mucosal epithelial cells in vitro.

Materials and methods: Oral mucosal epithelial cells were isolated and cultured in vitro. A 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to assess the viability of treated cells, while senescence was assessed by senescence-associated β-galactosidase staining. Cell surface marker expression was analyzed by flow cytometry. Finally, real-time quantitative polymerase chain reaction was performed to examine gene expression levels.

Results: Triphala extract (5 μg/mL) reversed the cell senescence activity of arecoline, as evidenced by reduced β-galactosidase activity, increased Ki-67 marker expression, and reduced expression of senescence-related genes p16 and p21.

Conclusion: Triphala extract helped to reduce the pathological effects of arecoline-induced pathogenesis. Clinical relevance. Arecoline found in the areca nut causes oral pathological conditions including oral submucous fibrosis. Our results showed that Triphala counteracted the adverse effects of arecoline, in particular, negating senescence in oral mucosal epithelial cells. As a translational effect, Triphala treatment could restore normal epithelial thickness in oral submucous fibrosis, thus reducing the clinical severity of the disease. This reestablishment of oral homeostasis would help to improve oral health-related quality of life in patients with oral submucous fibrosis.

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1. Introduction

Arecoline, an alkaloid found in the Areca catechu L species, exerts numerous pharmacological effects on the endocrine, cardiovascular, nervous, and digestive systems as well as serious toxic effects on oral cells. The main toxic effect of arecoline is caused by oxidative stress, which leads to cell death. Such toxicities have been linked to fibrosis and carcinoma (Khan et al., 2015; Liu et al., 2016; Thangjam et al., 2009). Upon exposure to arecoline, oral...
epithelial cells showed increased production of aldehyde dehydrogenase 1 (ALDH1), stemness, and epithelial-mesenchymal transition (EMT) characteristics (Wang et al., 2016). Rehman et al. showed that alkaloids from areca nuts induced senescence in oral epithelial cells and created a microenvironment, mainly owing to the overexpression of transforming growth factor-beta (TGF-β), which can cause malignancy. The levels of heat shock proteins (HSPs) such as HSP47, which are elevated in cancer, are also elevated in epithelial cells following exposure to arecoline (Lee et al., 2011).

The toxic effects of arecoline are not limited to epithelial cells. Endothelial cell damage has also been reported following exposure to alkaloid extracts, which leads to periodontitis and/or fibrosis (Tseng et al., 2012). Alkaloid exposure also caused endothelial cell cytotoxicity, contributing to reduced vascularity (Ullah et al., 2014). Moreover, Li et al. (Li et al., 2014) showed that arecoline induced apoptosis in epithelial cells, reducing their viability, which could explain the progression of oral fibrosis. While the molecular mechanisms underlying arecoline toxicity are not yet completely elucidated, the involvement of the Akt/mTOR signaling pathway could explain the progression of oral fibrosis. While the molecular mechanisms underlying arecoline toxicity are not yet completely elucidated, the involvement of the Akt/mTOR signaling pathway for the atrophy of oral epithelium.

We envisaged arecoline-mediated senescence as a novel proposition for the atrophy of oral epithelium.

Triphala is an ancient preparation, Rasayana or formulation in Indian medicine, comprising dried fruits from *Terminalia bellirica*, *Emblica officinalis*, and *Terminalia chebula*. This preparation is used as an antioxidant and for the treatment of fatigue and high-altitude polycythemia (Huang et al., 2018). Triphala has been shown to have radioprotective, chemoprotective, and anti-inflammatory properties (Sharma and Sharma, 2011; Sireeratawong et al., 2013) and helps in wound healing due to its antibacterial and antioxidant properties (Kumar et al., 2008). Triphala has also shown benefits in oral health by reducing plaque and gingivitis in adults and children (Baratakke et al., 2017; Bhattacharjee et al., 2015). However, its therapeutic properties require validation at the molecular level.

Previous studies have shown that arecoline induces senescence in oral epithelial cells (Rehman et al., 2016). In addition, p21 and p16 are markers of cell cycle arrest or cell senescence, while β-galactosidase is an enzyme that shows elevated levels in cells undergoing senescence (Terzi et al., 2016). Therefore, in the context of the beneficial properties of Triphala, we assessed its impact on arecoline-induced senescence oral epithelial cells by assessing markers such as p16, p21, and β-galactosidase (senescence assay).

2. Materials and methods

2.1. Preparation of Triphala extract and other reagents

Finely ground Triphala powder was obtained from a commercial source (Dabur India Ltd.: equivalent extents of *T. chebula*, *E. officinalis*, and *T. bellirica*, 1:1:1 w/w/w). The powder was weighed and soaked in purified water for 24 h in a conical flask at room temperature with constant shaking until the water was colorless. Particulate matter that did not dissolve was filtered using a syringe filter with a pore size of 0.22 μm. The remaining filtrate was lyophilized and weighed. A stock solution was prepared by mixing 100 mg extract in 1 mL water (100 mg/mL) and stored in the refrigerator for further experimental use.

Arecoline (Sigma-Aldrich, St. Louis, MO, USA) was obtained and mixed in purified water to make a final stock solution of 100 mM. For the experiments, the stock solution was directly added to the culture medium to reach the appropriate final concentrations.

2.2. Cell culture

Surgical biopsy samples were obtained from histopathologically normal oral mucosa during the surgical extraction of the third molars of six subjects aged 18–25 years after obtaining informed consent. Single-cell suspensions were prepared enzymatically using 0.25% trypsin-EDTA solution (Invitrogen, Carlsbad, CA, USA). The epithelial cells that were dissociated from the tissue were cultured and grown in keratinocyte growth medium (KGM-2, Lonza, Basel, CH) and incubated at 37 °C in 5% CO₂. All experiments used cells between the second and fourth passages.

2.3. Cell cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells were seeded in 96-well plates (1 × 10⁴ cells per well) and treated with various concentrations of the arecoline and Triphala (50 μM arecoline along with Triphala at 1, 2, 5, and 10 μg/mL). The IC50 values for Triphala and arecoline were 20 μg/mL and 100 μM, respectively (results not shown in this study). After 48 h of incubation, cytotoxicity was assessed by MTT assay at 570 nm on a microplate reader. Non-toxic concentrations were used for further experimental work.

2.4. Cellular senescence assay

The cells were seeded (1 × 10⁵ cells per well) into 6-well plates and incubated for 15 days at 37 °C. The cells were treated with arecoline (50 μM) and arecoline (50 μM) + Triphala (5 μg/mL) at each media change (every other day). After 15 days, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) and then subjected to senescence-associated β-galactosidase (SA-β-gal) staining according to the manufacturer instructions (Cell Signaling Technology, Danvers, MA, USA). The cells were then incubated overnight at 37 °C outside the incubator and protected from light. The stained cells were washed twice with PBS and examined under a light microscope.

2.5. Flow cytometry analysis of Ki-67 expression

The cells were harvested from the culture plates treated with the concentrations of interest and incubated with anti-Ki-67 antibody (FITC) (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation for 30 min at room temperature, the cells were analyzed on a flow cytometer (Attune NXT, Thermo Fisher Scientific, Waltham, MA). At least 10,000 events were gated for each sample. The percentages of positive cells were calculated for comparative analysis of each group.

2.6. Real-time quantitative polymerase chain reaction (RT-qPCR) for senescence-related genes

The cells were seeded (1 × 10⁵ cells per well) into a 6-well plate and incubated for 15 days at 37 °C. The cells were treated with arecoline (50 μM) and arecoline (50 μM) + Triphala (5 μg/mL) at...
each media change (every other day). After 15 days, the medium was removed, and the cells were washed twice with PBS. RNA extraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse transcribed using a cDNA synthesis kit (Invitrogen Life Technologies Carlsbad, CA, USA) according to the manufacturer’s instructions. Quantitative analysis of the genes of interest (p16 and p21) was performed using SYBR Green PCR master mix (Applied Biosystems, Austin, TX, USA) on a qRT-PCR system (QuantStudio 5, Applied Biosystems, Foster City, CA). Normalization of the expression of target genes to β-actin was performed using the ΔΔCt technique. The data were quantified using the 2−ΔΔCt technique and showed normalized relative gene expression to that of the average CT for the β-actin gene. A list of primers is shown in Table 1.

2.7. Statistical analyses

The data are presented as means ± standard deviations of the three independent experimental values. Statistical analyses were performed by one-way analysis of variance (ANOVA) om GraphPad Prism 8. p < 0.05 was considered statistically significant (*p < 0.05, **p < 0.01).

3. Results

3.1. Triphala extract at moderately higher concentrations (5 μg/mL) does not show any toxic effects in synergy with arecoline treatment and significantly increases oral mucosal epithelial cell viability

Oral mucosal epithelial cells treated with arecoline (50 μM) along with different concentrations of Triphala extract (1, 2, 5, and 10 μg/mL) were subjected to MTT assay to assess cell viability after 48 h of treatment. Arecoline treatment alone (50 μM) and arecoline (50 μM) and lower concentrations of Triphala (1 and 2 μg/mL) showed reduced cell viability compared to that in the control cells (Fig. 1A). However, cells treated with arecoline (50 μM) and a moderately higher concentration (5 μg/mL) of Triphala extract showed significantly higher viability than that of the arecoline treatment alone (50 μM) (Fig. 1A).

Examination by light microscopy showed that arecoline (50 μM) caused the cells to detach from the surface. The cells were floating in the culture medium and were sparsely distributed on the surface of the culture plates (Fig. 1B). However, the cells treated with arecoline (50 μM) and a moderately higher concentration (5 μg/mL) of Triphala extract were normal and were grown to confluence on the surface of the culture plates (Fig. 1B).

3.2. Treatment with Triphala extract significantly reduces the senescent effect of arecoline in oral mucosal epithelial cells

Senescence was assessed using an SA-β-Gal staining kit. Senescing cells release increased levels of β-galactosidase, which can be stained to evaluate the activity. Oral mucosal epithelial cells treated with arecoline (50 μM) showed increased β-galactosidase activity (Fig. 2A). However, the same (A50) cells treated with Triphala extract (5 μg/mL) showed significantly reduced β-galactosidase activity, as observed under the microscope (Fig. 2B) to count β-galactosidase-positive cells (Fig. 2A).

3.3. Triphala extract enhances the proliferative activity decreased by arecoline treatment and reduces the expression of senescence-related genes elevated by arecoline treatment

As observed previously, arecoline treatment affected cell viability. This treatment is also correlated with cell proliferative activity. The expression of Ki-67, a marker denoting the proliferative potential of cells, was reduced after arecoline treatment (50 μM), with a significantly higher Ki-67 expression level than those in cells treated with arecoline alone or in the control cells (Fig. 3A).

Table 1

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| p16  | 5'-ATG GAG TCC GCT GCA GAC AG-3' | 5'-ATG CCG GTA CGA CCC AAA G-3' |
| p21  | 5'-GGC GGC AGA AGA GCA TAG TAC CAG ACT-3' | 5'-GGC GGC GGC GGC CAG GCT AT-3' |
| ACTB | 5'-AGA GCT TGC AGC TGC CAG AC-3' | 5'-AGC ACT GTG TGC CAG TAC AG-3' |

Fig. 1. Single-cell suspensions were prepared from tissue samples by enzymatic digestion. The cells were then subjected to different treatments. (A) MTT assay to assess cell viability was performed in cells treated with arecoline (50 μM) and arecoline (50 μM) + Triphala extract (1, 2, 5, and 10 μg/mL). n.s. not significant, *p < 0.05, **p < 0.01 (B) Morphology of cells treated with arecoline (50 μM) and arecoline (50 μM) + Triphala extract (5 μg/mL). Scale bar = 100 μm.

4. Discussion

Increased expression of certain genes is a key characteristic of senescence. Senescence limits the cell doubling potential and also affects cell phenotypes. While treatment with arecoline (50 μM) resulted in significantly increased p16 and p21 gene expression levels, exposure to Triphala extract (5 μg/mL) significantly reversed...
Senescence (Arakeri et al., 2017; Ray et al., 2019; Tilakaratne et al., 2014; Rehman et al., 2016; Wang et al., 2016). The trophic awakening of senescence in oral mucosal cells and that this acquired phenotype could modulate the exposure of these cells to neoplastic transformation was noted. OSMF, and malignant transformation (Lee et al., 2011; Li et al., 2008). Chebulinic acid, a vital component of Triphala, attenuated vascular endothelial growth factor receptor-2 (VEGFR-2) phosphorylation to suppress angiogenesis and inhibit primary tumor growth and the potential for metastasis (Lu et al., 2012). Triphala also significantly inhibited primary tumor growth and metastatic potential in gastric carcinoma cells transplanted into a xenograft model of zebrafish by attenuating Akt, epidermal growth factor receptor (EGFR), and ERK phosphorylation (Tsering and Hu, 2018).

In normal cells, Triphala scavenges ROS and prevents oxidative damage. In contrast, in cancer cells, Triphala increases ROS levels and induces cell death (Vadde et al., 2015). Thus, the Triphala-induced attenuation of cellular senescence in the present study could indicate that the arecoline-treated oral mucosal cells did not undergo neoplastic transformation. If arecoline induces neoplastic transformation in oral mucosal cells, exposure to Triphala may lead to augmentation of senescence. Additional studies are needed in which oral mucosal cells in varying stages of neoplastic transformation are exposed to Triphala. The change in the effect of Triphala from the inhibition to the augmentation of senescence could be a critical point in neoplastic transformation.

The health-boosting characteristics of Triphala are generally ascribed to the polyphenolic secondary metabolites; namely, ellagic acid, chebulinic acid, and gallic acid present in Triphala extract (Baratakke et al., 2017; Bhattacharjee et al., 2015; Kumar et al., 2008; Sharma and Sharma, 2011). The major limitation of the present study was that it did not explore the molecular pathways of the Triphala effect countering arecoline-induced senescence. Elucidating these pathways could provide novel diagnostic and therapeutic targets. Thus, future studies are needed to explore at the molecular signaling and cellular biology levels the mechanisms of action of the effect of Triphala in reducing senescence as well as its other potential anti-carcinogenic effects.

5. Conclusion
The results of the present study simulated the clinical scenario to assess the direct action of arecoline on oral epithelial cells, as observed in OSMF. Treatment with Triphala extract increased the viability, reduced senescence-related gene expression, and enhanced the proliferation marker expression in oral mucosal epithelial cells treated with arecoline. As a translational effect, Triphala treatment could restore normal thickness in OSMF, thus reducing the clinical severity of the disease. Randomized controlled trials on the effect of Triphala on patients with OSMF are warranted. We also recommend exploring the effect of Triphala on other arecoline-induced, carcinogenesis-relevant signaling pathways.

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**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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