Effectiveness of alpha lipoic acid on oral squamous cell carcinoma

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ABSTRACT

Oral submucous fibrosis (OSF) is a chronic irreversible condition of the oral mucosa with proven malignant potential. However, till date, there has been a dearth of effective management strategies. This study used alpha lipoic acid, an antioxidant, in oral form to determine if it could be useful in managing OSF patients. A case-control study was conducted on 18 patients of OSF (9 cases and 9 controls). The case group was treated using alpha lipoic acid in addition to the intralesional steroid and hyaluronidase injections used in the control group. The cases in the alpha lipoic acid group exhibited better relief of symptoms such as burning sensation of the mucosa and mouth opening, as compared to the controls. The use of alpha lipoic acid along with intralesional steroids and hyaluronidase caused reversal of higher clinical stages to lower ones. The use of an antioxidant, alpha lipoic acid, along with conventional therapy of intralesional steroid injections definitely seems to have a beneficial impact in the management of OSF.

Keywords: alpha – stages-antioxidants-OSF

Introduction

Oral cancer is one of the serious and growing health problems worldwide and oropharyngeal cancer is a significant component of the global burden of cancer. The annual estimated incidence is around 275,000 for oral and 130,300 for pharyngeal cancers, two-thirds of these cases occurring in developing countries.[1,2] Oral cancer ranks in the top three of all cancers in India which account for over 30% of all cancers reported in the country.[3] Treatment modalitiesof oral squamous cell carcinoma (OSCC) have numerous side e ects. Clinical consequences of radiotherapy include mucositis, oral candidiasis, loss of taste, and xerostomia, which may be permanent due to the detrimental e ect of radiation on salivary glands.[4] Despite developments in current treatment modalities using chemotherapy , surgery and radiation, along with other palliative q, OSCCremains a great challenge for clinical therapy. Accordingly, new strategies are evolving to control and to treat cancer and one such strategy could be the use of medicinal plants. Recent studies have been focused on herbal medicine as potent anti-cancer drug candidates.[5]

This study was conducted because there was limited studies that was used to detect the anti cancer effect of ALA. They are widely used in other countries for the treatement modalities for carcinoma except of oral squamous cell carcinoma.(6)

MATERIALS AND METHODS

Chemicals

α – lipoic acid, trypan blue solution (0.4%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. India. The other chemicals used in this study were purchased locally and were of analar grade.

Cell culture

The SCC-25 human oral squamous carcinoma cell line was procured from ATCC. Cells were maintained in Dulbecco's Minimum Essential Media and Ham's F-12 (1:1 ratio) supplemented with 10% fetal bovine serum (FBS), with 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Cells were grown in 75 cm^2 culture flasks and after a few passages, cells were seeded for experiments. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using 0.05% Trypsin-EDTA solution.

Cell treatment

α – lipoic acid was dissolved in 0.1% DMSO (v/v). SCC-25 cells were plated at 10,000 cells/cm^2. After 24 h, cells were fed with fresh expansion culture medium supplemented with different final concentrations of ALA (25 and 50 μg/mL) or the corresponding volumes of the vehicle. The ALA concentration used in this study was selected based on the evaluation of IC_{50} concentration. After 24 h of treatment, cells were collected after trypsin application. Total cell number was determined by counting each sample in triplicate under inverted microscope. Viability was also evaluated by the trypan blue dye exclusion assay.

MTT assay

Cytotoxic effect of ALA on SCC-25 cells was assessed by MTT assay Safadi *et al.,*[18] using concentrations of 0.1–1000 μg/mL for 24 h. Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well. After 24 h, cells were fed with fresh expansion culture medium supplemented with different final concentrations of ALA (0.1-1000 μg/well) and incubated for 24 h. Untreated cells served as control and received only 0.1% DMSO. At the end of treatment period, media from control, ALA-treated cells was discarded and 50 μL of MTT (5 mg/mL of phosphate-buffer saline (PBS)) was added to each well. Cells were then incubated for 4 h at 37° C in CO_2 incubator. MTT was then discarded and the coloured crystals of produced formazan were dissolved in 150 μL of DMSO and mixed effectively. The purple blue formazan dye formed was measured using an ELISA reader (BIORAD) at 570 nm.

Acridine orange/ethidium bromide (Dual staining)

Acridine Orange/Ethidium Bromide orange staining was carried out by the method of Gohel *et al.,*[19] SCC-25 cells were plated at a density of 1×10^4 in 48-well plates. They were allowed to grow until they are 70–80% confluent. After 24 h the cells were treated with 25 and 50 μg/mL of ALA for 24 h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and ALA treated were mixed with 100 μL of dye mixture (1:1) of ethidium bromide and acridine orange and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification. A minimum of 300 cells was counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

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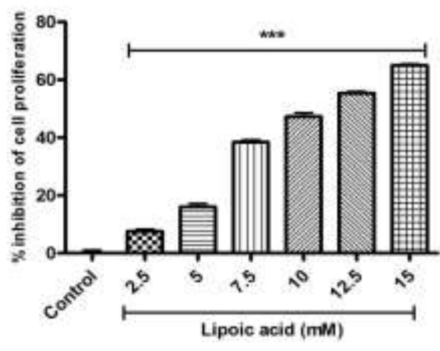
Cell cycle arrest analysis by flow cytometry

To investigate the effect of ALA on the cell cycle distribution, SCC-25 cells (1×10^5 cells/mL) were treated with 25 and 50 μg/mL of ALA for 24 h. The treated cells were harvested, washed with PBS and fixed in 75% ethanolic at 4° C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 μg/mL propidium iodide (PI) and 0.1 mg/mL RNase A followed by shaking at 37° C for 30

min. The stained cells were analysed with flow cytometer (Becton-Dickinson San Jose, CA, USA) and the data were consequently calculated using WinMDI 2.9 software (TSRI, La Jolla, CA, USA) (Tu et al. 2004).
Statistical analysis

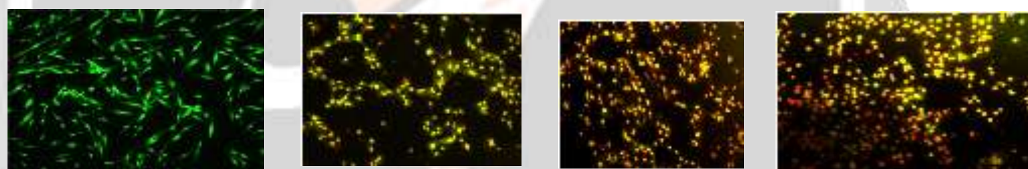
Data were expressed as mean ± S.E.M and analysed by Tukey's test to determine the significance of differences between groups. A *p* value < 0.05, 0.01 or/and 0.001 was considered to be significant.

RESULTS



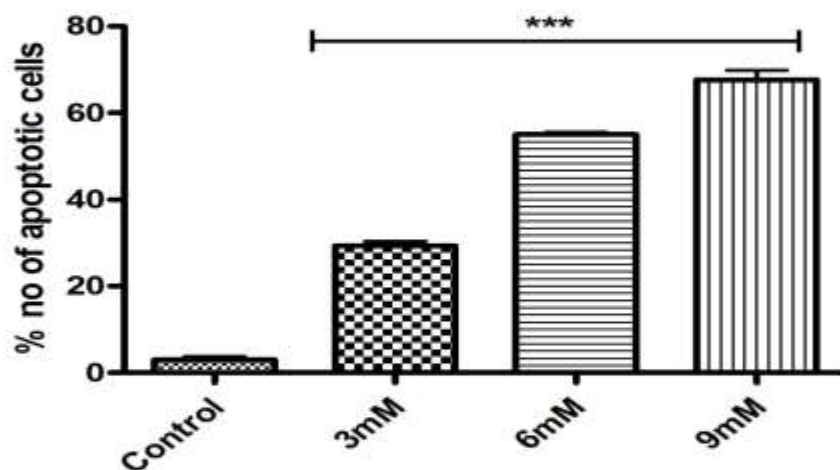
Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed by One way ANOVA followed by Dunnett's test. P value ≤ *0.05, **0.01 and ***0.001 considered significant

Representative images of SCC - 25 control and Lipoic acid treated cells stained with ethidium bromide and acridi



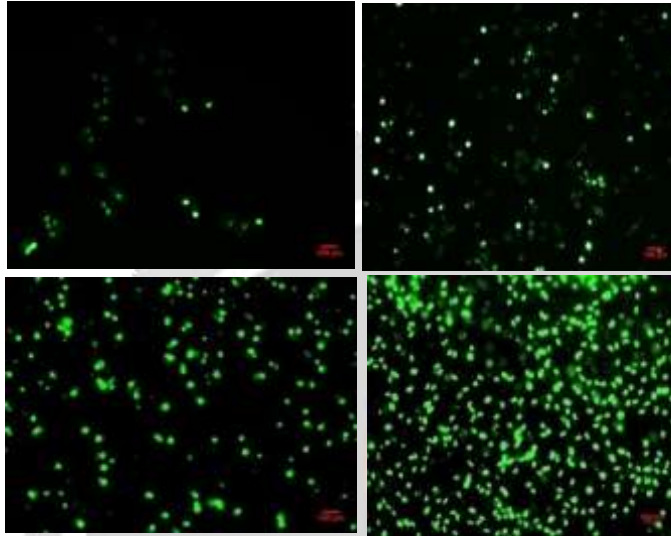
Control. 3Mm. 6Mm. 9 Mm

Bar graph showing the percentage number of apoptotic cells stained using Ethidium bromide – acridine orange stain



Values are expressed as mean ± SEM (n=3). Apoptotic cells were individually calculated as percentage of apoptotic cells relative to the total number of cells in each random field and represented the average of three independent experiments ± SEM. Statistical analysis was performed using Graph Pad prism 5.0. Comparison as done using one way ANOVA

Effect of Lipoic acid on ROS inhibition SCC – 25 cancer cells - Dichlorofluorescein staining assay



Lipoic acid treatment exhibited induction of apoptosis in SCC – 25 cancer cells. The percentage number of apoptotic cells was significantly increased on concentration dependent manner

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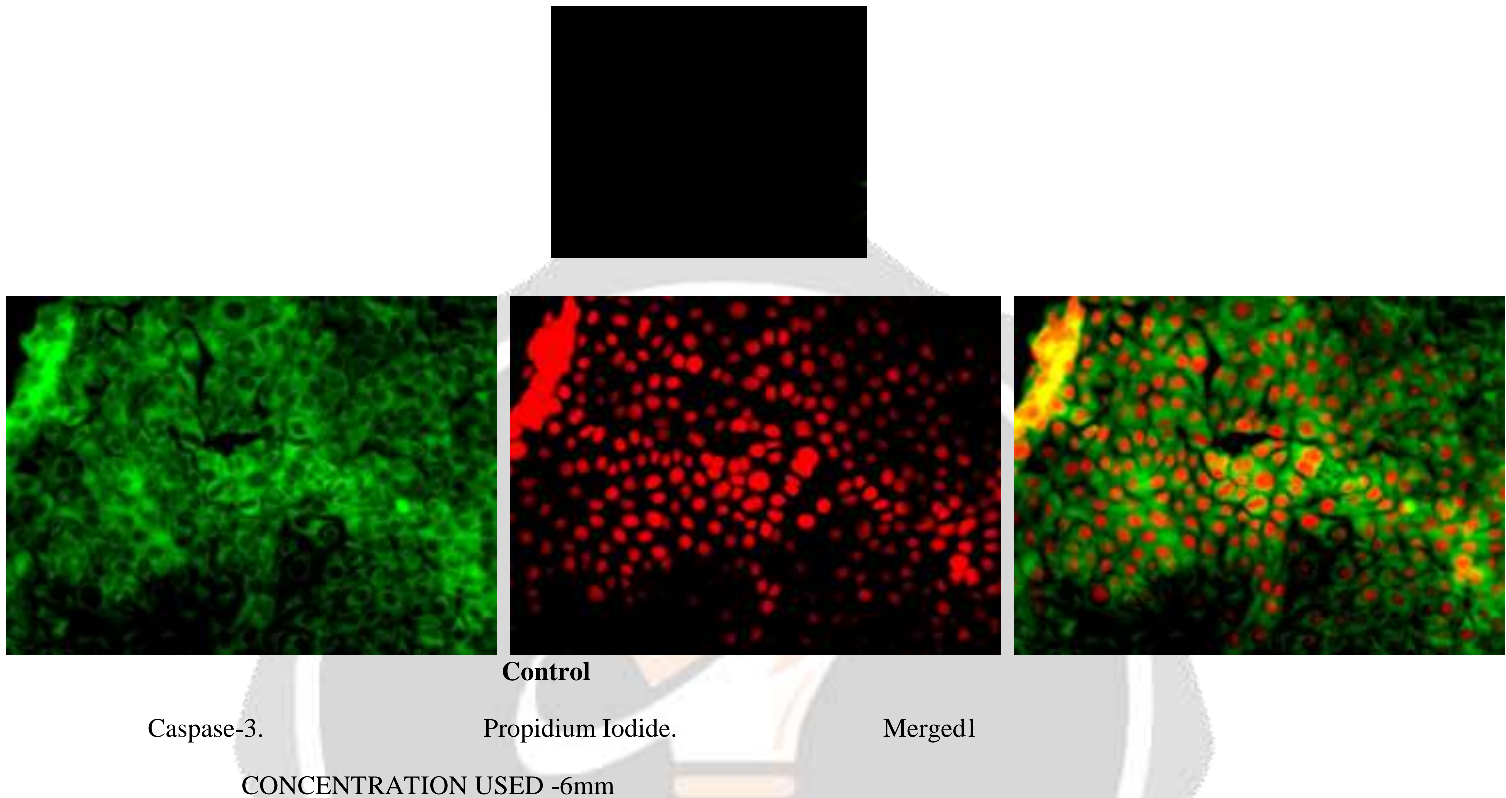
Effect of Lipoic acid (IC50 concentration) on Caspase 3 activity in SCC – 25 cancer cells - Double Immunofluorescence assay



Control

Caspase-3. Propidium Iodide. Merged1

CONCENTRATION USED -6mm

- **Double immunofluorescent staining to Caspase 3 of SCC – 25 oral cancer cells. Propidium iodide was used as nuclear counter stain**
- **Increased Caspase 3 expression was observed in 6mM (IC50) Lipoic acid treated cells**

Effect of Alpha lipoic acid on the proliferation of OSCC cells

We evaluated the cytotoxic effect of alpha lipoic acid in OSCC cells. The morphology of OSCC cells treated with alpha lipoic acid is depicted in Figure 1A. Initially, we selected broad concentration ranges (3,6,9 Mm) to find out the effective concentrations in OSCC cells. The Alpha lipoic acid treatment for 24 h significantly ($P < 0.001$) inhibits the proliferation of OSCC cells (Figure 1B).

Effect of Alpha lipoic acid on the plasma membrane translocation of phosphodityl serine

In order to find out the exact mechanism behind the GF induced decrease in proliferation, we evaluated the plasma membrane translocation of alpha-lipoic acid by fluorescein-labelled annexin V staining. Alpha lipoic acid treatment (3,6 and 9 Mm) caused apoptosis from the inner side of the plasma membrane to the surface. The intensity of annexin V immunofluorescence is more positive in the experimental groups when compared to control (Figure 2).

Analysis of apoptosis by Ethidium bromide and Acridine orange staining

Ethidium bromide and acridine orange staining was also used to identify apoptotic cells by fluorescence microscopy. Ethidium bromide is a protein that is conjugated to a green florescent dye to detect apoptosis. Acridine orange is a red fluorescent dye that stains DNA of both necrotic and late apoptotic cells with damaged membranes. Alpha lipoic acid treated cells were positive to Ethidium bromide and Acridine orange indicating the presence of early and late apoptotic cells. The control cells were viable and hence negative to Ethidium bromide and Acridine orange (Figure 3).

Apoptotic nuclear morphology by Dual staining

Apoptotic nuclear morphology was observed after Dual staining using fluorescence microscopy. After treatment with different concentrations of GF for 24 h, OSCC cells began to exhibit apoptotic characteristics, such as cell shrinkage, nuclear condensation, and fragmentation. While the cells observed in the control group did not show any significant changes in the nuclear morphology (Figure 4).

Alpha lipoic acid effect on the p53 gene and protein expressions

We further investigated the changes in the tumor suppressor p53 expression at gene and protein level. Alpha lipoic acid treatment caused significant up regulation of p53 expression at gene and protein level than that of control. The high expression of p53 observed with the maximum Lipoic acid concentration used in this stduy 6 mm.. GAPDH and β-actin is used for normalization of p53 gene and protein respectively (Figure 5A-D).

DISCUSSION

There are several hypothesis regarding the mechanism of action by which ALA may prove that it can be useful in the treatement of cancer. ALA is used in the various type of carcinoma except oral squamous cell carcinoma . It is the agent of more anti- cancer property. Numerous preclinical study looking for the use of ALA alone or in combination with other agents for the treatement of cancer, the human intervention literature is limited. There are a small number of randomised and non- randomised trial and a few case in the use of ALA in the treatement of chemotherapy. And hence this study was conducted to view its effects on OSCC.

Ethidium Bromide and Acridine orange staining is a commonly used method for qualitatively evaluating the apoptotic cells. Ethidium bromide binds to phosphatidylserine (PS) exposedl on the outer membrane leaflets of apoptotic cells also appears on the surfaces of dead cells. In addition, the characteristics of late apoptosis include some loss of membrane integrity and Ethidium bromide to pass through the membranes, intercalate into nucleic acids, and display red fluorescence (Baskić et al., 2006; Rieger et al., 2011). In view of the above reports, the Ethidium bromide and Acridine orange stained cells in experimental groups indicating the fact that OSCC cells are in late apoptotic stage.

It was reported the staining is not suitable for the evaluation of early apoptotic cells (Rieger et al., 2011) and hence; we examined the early apoptotic cells through plasma membrane translocation of PS. Translocation of membrane PS from the inner side of the plasma membrane to the surface is reported one of the earlier events of apoptosis (Segawa and Nagata, 2015). ALA treatment induced significant translocation of phosphatidylserine to periphery of OSCC cells indicating strong evidence of early

apoptosis. The morphological changes characteristic of cell death were also investigated by dual staining. This staining is usually performed to investigate the nuclear damage upon apoptosis induction or DNA damage in cancer cells (Zhang et al., 2013). As confirmation of apoptosis, the morphological changes characteristic of apoptotic nuclei of ALA treated OSCCcell was the appearance of nuclear fragments and condensed chromatin when compared with

untreated cells. These results further correlates with the above findings.

Therefore, we analyzed the morphology of ALA treated OSCC cells with AO/EB dual staining. Syringic acid treatments for 24 h caused significant alterations in the morphology of OSCC cells. And there was no significant change detected in the negative control group. Early apoptotic cells, marked yellow-green AO nuclear staining, were detected in the low dose of ALA treatment (3Mm). While late apoptotic cells localized with red nuclear EB staining were detected in the all experimental groups except control (Fig. 3A). The quantification of apoptotic cells further conformed by the dose-dependent increase of apoptotic cells in experimental groups than that of control (Fig. 3B). Studies have reported that EB stain only entered cells with damaged membranes, such as late apoptotic and dead cells, emitting red fluorescence when bound to concentrated DNA fragments or apoptotic bodies (Liu et al., 2015; Curčić et al., 2012) [27,28]. nic

The p53 tumor suppressor regulates cell cycle progression and cell survival in response to cellular stress (Ezhilarasan et al., 2017). This protein is also responsible for the regulation of the senescence of cells and the cell entering and leaving the subsequent stages of the cellular cycle (Rufini et al., 2013). DNA damage or oncogenic stress induces p53 protein levels, allowing elimination of incipient tumor cells by apoptosis. When DNA damages are induced, the cell cycle is arrested and p53 is activated for DNA repair (Williams and Schumacher, 2016). If DNA repair is not successful, then p53 causes apoptosis by induction of Bax (Zhao et al., 2017). In this study, ALA treatment caused significant increase in the levels of p53 expression both in gene and protein level which could be probably due to the apoptosis induction in OSCC Cells. It is reported that p53 participates directly in the intrinsic apoptosis pathway by interacting with the multidomain members of the Bcl-2 family to induce mitochondrial outer membrane permeabilization (Gudkov and Komarova, 2016). The overall finding of the present study suggests that ALA treated OSCC cells i) the externalization of PS at early stages after the induction of apoptosis shown by immunofluorescence ii) late apoptosis and necroptosis as shown by Ethidium bromide and Acridine orange iii) nuclear fragmentation and condensation as shown by DUAL staining iv) activation of p53 gene and protein expression. These findings undoubtedly confirm the induction of apoptosis in OSCCcells upon ALA treatment.

CONCLUSION

These recent findings suggest that ALA may have a potent anti- proliferative effects on Oral squamous cell carcinoma with the induction of apoptosis.ALA is likely to be valuable for the management of OSCC

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