The Synergistic Cytotoxic Effect of Low-Level Laser Therapy and 5-Aminolevulinic Acid-Mediated Photodynamic Therapy on Tongue Squamous Cell Carcinoma Cell Line

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Original Article

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ABSTRACT

Introduction: Nowadays, the global burden of cancer has grown substantially, endangering the public health. Thus, the discovery of innovative therapeutic modalities with the fewest adverse effects that improve patient quality of life has become compulsory. Research about the significance of utilizing photodynamic therapy (PDT) and enhancing its effect in neoplasms treatment has become crucial to fully comprehend its impact on neoplastic cells of various origins before adopting it into clinical practice.

Aim of the Work: The current study aimed at investigating the cytotoxic effect of 5-aminolevulinic acid-mediated photodynamic therapy (5-ALA-PDT) on human tongue squamous cell carcinoma (HNO-97) cells and assessing the possible enhancement of its efficacy by combining it with low-level laser therapy (LLLT).

Material and Methods: The current study was divided into five groups; control group, 5-ALA only group, LLLT only group, 5-ALA-PDT group and a combination of LLLT and 5-ALA-PDT group. Two concentrations of 5-ALA were utilized throughout the experiment. To investigate the effects of the various treatments, the evaluation was done utilizing MTT cytotoxicity assay to assess the viability of different treatments, DNA fragmentation assay to evaluate apoptosis qualitatively, examination of cytological alterations by H&E stain and Ann-V/PI double staining to differentiate between different types of cell death. Statistical analysis was done using ANOVA, multiple comparisons post hoc Tukey test and unpaired t-test for each variable of the results.

Results: MTT assay results showed a significant decrease in the mean viability percentages of all studied groups and subgroups in comparison to the untreated negative control subgroup and a non-significant decline of that of the combination treatment subgroups when compared to 5-ALA-PDT subgroups. DNA fragmentation, cytological examination and Ann-V/PI results revealed an increase in the apoptosis rate across all treatment groups.

Conclusion: 5-ALA-PDT had an obvious cytotoxic effect on HNO-97 cells that can be further reinforced by combining it with LLLT.

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Key Words: 5-aminolevulinic acid; low-level laser therapies; photodynamic therapy; photobiomodulation therapies; tongue cancer.

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INTRODUCTION

Cancer is a significant public health concern on a global scale. Worldwide, it is considered the second root cause of fatality following ischemic heart disease accounting for annual 9 million deaths[1]. Oral tongue squamous cell carcinoma (OTSCC) is considered one of the most deleterious neoplasms of the oral cavity and it accounts for 20% of head and neck cancers being one of the most frequently occurring neoplasms of this group of malignancies[2,3]. OTSCC survival rate is still low owing to the grievous side effects of the conventional therapeutic modalities. The emergence of innovative treatment modalities specifically targeting neoplastic cells with minimal adverse effects has thus been an uprising demand recently[4,5].

Photodynamic therapy (PDT) has gained applause recently as it is considered a minimally invasive therapeutic approach selectively targeting neoplastic cells and thus saving the patients from the deleterious effects of standard treatment options. It encompasses the interplay of a photosensitizer (PS), a source of light and the availability of tissue oxygen which triggers the generation of toxic reactive oxygen species (ROS) that end up in apoptosis or necrosis of neoplastic cells. Of particular, 5-aminolevulinic

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acid-mediated photodynamic therapy (5-ALA-PDT) is one of the most promising research topics in the field of development of PDT for cancer treatments as 5-ALA is an exogenous precursor of a second-generation endogenous photosensitizer that is generated from the heme synthesis pathway, named protoporphyrin IX (PpIX). PpIX aggregates in highly metabolic cells such as neoplastic cells due to the absence of ferrochelatase enzyme as well as reinforced activity of porphobilinogen deaminase enzyme leading to the production of significant amounts of ROS and ending up in cell death[6].

Another light/laser application that has made significant strides in the last decade is low-level laser therapy (LLLT), also referred to as photobiomodulation, (PBM). It utilizes light photons (wavelengths ranging from 390-1100 nm and fluences ranging from 0.04 to 50 J/cm²) to alter biological processes in the cells[7,8]. Biphasic dose-response, alternatively referred to as the hormesis phenomenon, indicates that in terms of fluence, irradiance, illumination time or application number, extremely low as well as extremely high doses of light or laser energy may have insignificant impacts[9,10]. This concept applied to LLLT implies that utilizing laser in reduced power densities results in biostimulatory effects whereas greater power densities entail inhibition of cell metabolism[11].

Thus, this study was conducted to assess the cytotoxic effect of 5-ALA-PDT and investigate the isolated effect of applying low-level laser (LLL) on tongue squamous cell carcinoma cell line (HNO-97). Also, in an attempt to enhance the 5-ALA-PDT effect, this study assessed the potentiality of combining LLLT and 5-ALA-PDT to enhance the anticancerous effect of 5-ALA-PDT on HNO-97 by pre-radiating HNO-97 cells with LLL before the application of 5-ALA-PDT.

MATERIAL AND METHODS

Cell Culture

Human tongue squamous cell carcinoma cell line (HNO-97) (CLS GmbH, Eppelheim, Germany) was supplied, established by successive sub-culturing, then treated according to all treatment protocols performed in this study at the Pharmacology Unit, National Cancer Institute, Cairo University, Egypt. HNO-97 cells were cultivated and maintained in sterile flasks with Dulbecco Modified Eagle's Medium (Gibco, Thermo Fisher Scientific Inc., USA) supplied with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin and kept in the dark at a pH of 7.2 in temperature-controlled incubators (Forma™ Series II Water-Jacketed CO_2 Incubators, Thermo Fisher Scientific Inc., USA) at 37 ºC and atmosphere of 95% air / 5% CO_2. Trypsin (Gibco, Thermo Fisher Scientific Inc., USA) as well as Phosphate buffered saline (PBS) (Sigma Aldrich- USA) were used in harvesting and passaging of the cells.

Pilot Experiment

A pilot experiment was conducted to identify the best irradiation time as well as the output power of the employed diode laser in the PDT and LLLT protocols of this study resulting in the most optimal results. All groups were treated following the PDT protocol suggested by Shinoda *et al*. 2021 and were illuminated with a 10 J/cm² laser beam but with different output power and irradiation time[12]. HNO-97 cells were incubated 24 hours post-irradiation step of PDT and then MTT cytotoxicity assay was performed to determine the viability of each group. The results showed that laser beams of higher power and shorter time of irradiation yielded the best outcomes. Hence, the laser device employed in this study was operated at its highest available power.

Different Treatments of HNO-97 Cells

For all different treatments of HNO-97 cells, cells were cultured in 96-well flat-bottom microplates for MTT cytotoxicity assay, whereas they were cultivated in 12 well cell culture plates to be harvested for the other different assessments. After different treatments were performed, cells were further incubated for 24 hours before different assessments were conducted.

Control Group

HNO-97 cells of the positive control subgroup were treated with IC50 concentration of Cisplatin (Mylan Company, France) which was obtained in a vial form of 1 mg/ml (subgroup Cis), whereas, the untreated HNO-97 cells of the negative control subgroup (subgroup C) were harvested with culture media and methanol only.

LLLT (L Group)

A dental diode laser device (LX16 Plus, Guilin Woodpecker® Medical Instrument Co., Ltd, China) of red wavelength (650 nm) was employed in the LLLT as well as PDT treatments. In order to evaluate the isolated effect of LLL, untreated HNO-97 cells were irradiated with the same laser parameters (650 nm of 10 J/ cm² fluence for 190 seconds) employed in the irradiation step of PDT.

5-ALA (A Group)

5-aminolevulinic acid hydrochloride (Sigma Aldrich – USA) obtained in a powder form (≥ 98% purity) was dissolved in dimethyl sulfoxide (DMSO) to be ready for use. HNO-97 cells were incubated with two different 5-ALA concentrations (subgroup A1: half IC50 concentration, subgroup A2: IC50 concentration) for 4 hours in the dark in a humidified incubator (37 ºC and 95% air / 5% CO_2 atmosphere). Cells were then washed twice with PBS and the culture medium was replaced with a fresh one.

5-ALA-PDT (P Group)

HNO-97 cells were treated following the protocol suggested by Shinoda *et al*. 2021 with different concentrations of 5-ALA (subgroup P1: half IC50 concentration, subgroup P2: IC50 concentration) for 4 hours in the dark in a humidified incubator[12]. Then, cells were washed twice with PBS and a fresh culture medium

was added. Post-incubation, cells were exposed to a 650 nm laser beam of 10 J/cm^2 fluence. Laser beam parameters were set to 200 mW output power and 190 seconds irradiation time. As per Shinoda *et al*. recommendation and the adoption of the same range of laser beam wavelength by other research groups (around 635 nm) for PpIX excitation, a 650 nm wavelength laser beam was utilized throughout the experiment for irradiation[12-15].

Combination of LLLT and PDT Group (LP Group)

HNO-97 cells were first irradiated with a 650 nm laser beam of 10 J/ cm^2. Then, these cells were treated following the same previously mentioned treatment protocol of 5-ALA-PDT (subgroup LP1: half IC50 concentration of 5-ALA, subgroup LP2: IC50 concentration).

MTT Cytotoxicity Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity assay kit (Sigma Aldrich, USA) was utilized for determining the IC50 concentration of 5-ALA and Cisplatin as well as for evaluating the viability of cells in different study groups and subgroups. MTT assay was conducted at the Pharmacology Unit, National Cancer Institute, Cairo University, Egypt.

For determining the IC50 of 5-ALA and Cisplatin, cells were incubated with a series of concentrations of 5-ALA and Cisplatin for 4 and 24 hours respectively. Whereas for assessment of the viability of cells in different study groups and subgroups, the cells were treated according to the previously mentioned treatment protocols, followed by incubation for 24 hours at 37°C in a humidified incubator thereafter. Post-incubation, the culture medium was replaced with a fresh one and MTT solution was added to the wells and incubated for 4 hours in a humidified incubator. Then, the medium was decanted, DMSO was added to the wells to dissolve the formed formazan crystals and the well plates were reincubated again for 30 minutes. Finally, the intensity of the produced purple color of the dissolved formazan crystals was quantified with the aid of an ELISA microplate reader (Infinite M Nano Tecan Sunrise Reader, Germany) at 540 nm. The viability percentage of cells was calculated according to the following equation:

Cell viability (%) = (Mean ofAbsorbance of treated cells)/ (Mean of Absorbance of control untreated cells) ×100[16]

Mean viability percentages produced from the serial concentrations of 5-ALA and Cisplatin used to calculate the IC50 of 5-ALA and Cisplatin are summarized in (Table 1).

Table 1: Mean viability percentages of MTT assay used in calculating the IC50 of 5-ALA and Cisplatin

	5-ALA concentration				
	0 mM	0.375 mM	0.750 mM	1.500 mM	3.000 mM
Viability %	100	72.4	59.7	47.7	36.5
	Cisplatin Concentration				
	0 µM	25 µM	50 µM	100 µM	200 µM
Viability %	100	91.4	75.3	54.1	49.9

The MTT cytotoxicity assay results revealed that the IC50 of 5-ALA was 1.35 mM and that of Cisplatin was 177 uM.

Gel Electrophoresis Assay for Analysis of DNA Fragmentation

DNA fragmentation analysis by agarose gel electrophoresis was conducted at Alroaa Laboratory (Cairo, Egypt). DNA extraction, Zymoresearch Quick-gDNA™ MiniPrep kit (Zymo Research Corp, Orange, California, USA) was employed in the DNA extraction step as per the manufacturer's instructions.

Extracted DNA was run at 100 Volts, 250 Amperes for 20 minutes. Then, DNA bands were visualized in relation to a 100 bp DNA ladder with the aid of a gel documentation system (BioDocAnalyze, Biometra, Goettingen, Germany).

Cytological Assessment

Steps of cytological assessment including H&E staining, examination as well as imaging were performed in the Precision Measurement and Tumor Markers Unit, Oral Pathology Department, Faculty of Dentistry, Ain Shams University.

Ten different microscopic fields showing apoptotic and necrotic cells were selected at random for imaging at an original magnification of 100X (Oil immersion) using a digital camera (EOS 650D, Canon, Japan) mounted on a light microscope (BX60, Olympus, Japan).

Annexin-V/Propidium Iodide (Ann-V/PI) Double Staining

Annexin-V and Propidium Iodide (PI) double staining assay was carried on at Global Research Labs, Cairo, Egypt. It was performed utilizing Alexa Fluor 488 Annexin-V/ Dead Cell Apoptosis Kit (In*vitro*gen, ThermoFisher Scientific, USA).

A 16 MP LaboMed Atlas Microscope Digital Camera mounted to a LABOMED Fluorescent microscope (LX400, Labomed Inc., California, USA) was employed to examine and image the stained cells. Two filters (FITC filter of 495/519 nm for Ann-V and Tetramethyl Rhodamine (TRITC) filter of 535/617 nm for PI) were used to examine and photomicrograph microscopic fields at an original magnification of 40X. Photomicrography as well as determination of the positively stained cells percentage were done with the aid of Pixel Pro 3.0 software (Labomed Inc., California, USA).

Statistical Analysis

The statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, New York, USA) was used to code and input the data gained from the results. Data were summarized as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) and multiple comparisons post hoc Tukey test were utilized to compare all studied groups and subgroups, whereas unpaired t-test

was used in comparing every two subgroups of the same group. *p-values* of different test results less than 0.05 were considered statistically significant[17].

RESULTS

MTT Cytotoxicity Assay Results

MTT cytotoxicity assay was employed in the current study to determine the viability percentage of all studied groups and subgroups. Statistical results of mean viability percentages of all studied groups and subgroups are summarized in (Table 2) and demonstrated in (Figure 1).

As compared to subgroup C and group L, the results revealed a steady decline in the mean viability percentages of the different treatment groups of HNO-97 cells treated with the same concentration of 5-ALA, the lowest mean viability percentage was seen in the LP2 subgroup. ANOVA test results revealed a high statistically significant difference on comparing the mean viability percentages of all studied groups and subgroups.

Post-Hoc multiple comparison test results showed a high statistically significant difference on comparing the mean viability percentage of subgroup C with those of all other studied groups and subgroups (*p-value* with group L= 0.007, *p-values* with all other subgroups =0.000). A statistically non-significant difference was detected on comparing the mean viability percentage of subgroup P1 with that of subgroup LP1 (*p-value* = 0.969) as well as on comparing the mean viability percentage of subgroup P2 with that of subgroup LP2 (*p-value* = 0.207). Additionally, a high statistically significant difference was revealed on comparing the mean viability percentage of subgroup Cis with those of subgroup C, group L, subgroups A1 as well as LP2 (*p-values* = 0.000-0.001), whereas a statistically non-significant difference was detected on comparison with those of subgroups P1, LP1, A2 and P2 (*p-values* > 0.05).

Additionally, results revealed that across all treatment groups, a decline in the mean viability percentage was noted on comparing the subgroups treated with half IC50 concentration of 5-ALA (A1, P1, LP1) when compared to those of subgroups treated with IC50 concentration of 5-ALA (A2, P2, LP2) of the same treatment group. This was further confirmed with unpaired t-test results comparing every two subgroups of the same group, which revealed high statistically significant differences between the mean viability percentages of subgroups C and Cis, subgroups A1 and A2, subgroups P1 and P2 as well as subgroup LP1 and LP2.

Gel Electrophoresis Assay Results

Since the hallmark of apoptosis is internucleosomal DNA fragmentation, qualitative evaluation of HNO-97 cells apoptosis of all treatment groups and subgroups in comparison to C and Cis control subgroups was done with the aid of analyzing DNA fragmentation and laddering by gel electrophoresis. The results revealed no DNA laddering in both subgroup C as well as group L, whereas, evident DNA laddering was observed in all other studied groups and subgroups as demonstrated in (Figure 2).

Among the subgroups treated with half IC50 concentrations of 5-ALA, DNA laddering of subgroup LP1 was more conspicuous compared to subgroups A1 and P1. Additionally, DNA laddering was more pronounced in the subgroups treated with IC50 concentration when compared to their corresponding treated with half IC50 concentration. Among all studied groups and subgroups, LP2 and Cis subgroups showed the most prominent DNA laddering.

Cytological Assessment Results

Our cytologic assessment results further validated the MTT cytotoxicity assay results. This assessment aimed at distinguishing between early apoptosis, late apoptosis and necrosis morphologic changes. The morphologic changes associated with early apoptosis are cell shrinkage, cytoplasmic membrane blebbing as well as chromatin margination, whereas those of late apoptosis are fragmentation of the nucleus and break down of cells into apoptotic bodies carrying nuclear fragments. With regards to necrosis, morphologic features include cell swelling, karyolysis or karyorrhexis and loss of plasma membrane integrity. HNO-97 cells of subgroup C exhibited regular morphology with some signs of pleomorphism with scarce apoptotic and necrotic cells. In group L, a minor number of apoptotic cells as well as scanty necrotic cells were detected. Cytologic examination revealed an increase in the number of apoptotic HNO-97 cells among different treatment groups (A group < P group < LP group) at half IC50 and IC50 concentrations of 5-ALA in relation to subgroup C and group L. Similarly, the number of necrotic cells increased among the different treatment groups following the same pattern but to a much lesser extent. Regarding subgroup Cis, numerous apoptotic cells as well as a few necrotic cells were detected. Cytological assessment images of all studied groups and subgroups are shown in (Figure 3).

Annexin-V/Propidium Iodide (Ann-V/PI) Double Staining Results

The percentages of early apoptotic, late apoptotic as well as necrotic cells of all studied groups and subgroups were determined using Annexin-V/Propidium Iodide (Ann-V/PI) double staining. Statistical results of mean percentages of early apoptosis, late apoptosis, total apoptosis and necrosis of all studied groups and subgroups are summarized in (Table 3 and illustrated in Figure 4).

Regarding apoptosis, the results showed a progressive increase in the total mean percentages of apoptosis of all treatment subgroups treated with the same concentration of 5-ALA (A1 < P1 < LP1 and A2 < P2 < LP2) in comparison to subgroup C and group L. Additionally, the ANOVA test showed a high statistically significant difference in the total mean percentage of apoptosis among all studied

groups and subgroups. Post-hoc multiple comparison test results revealed a statistically non-significant difference on comparing the total mean percentage of apoptosis of subgroup C with that of group L (*p-value* = 0.611), whereas on comparison with those of all other studied subgroups, a high statistically significant difference was detected (*p-value* = 0.000). On comparing the total mean percentage of apoptosis of subgroup P1 with that of subgroup LP1, a high statistically significant difference was detected (*p-value* = 0.001). On the contrary, on comparing the total mean percentage of apoptosis of subgroup P2 with that of subgroup LP2, a statistically non-significant difference (*p-value* = 0.611) was revealed.

Moreover, the results showed an increase in the total mean percentages of apoptosis in subgroups treated with IC50 concentration (A2, P2, LP2) when compared to their corresponding subgroup treated with half IC50 concentration (A1, P1, LP1) and according to the unpaired t-test results, this was statistically significant across all treatment groups; subgroup A1 and subgroup A2, subgroup P1 and P2, as well as subgroup LP1 and LP2. Also, a high statistically significant difference was detected on comparing subgroup C with subgroup Cis.

Referring to necrosis, the results followed the same pattern of apoptosis but to a much lesser extent where a gradual increase in the mean percentages of necrosis was detected across all treatment subgroups treated with the same concentration of 5-ALA (A1 < P1 < LP1 and A2 < P2 < LP2) when compared to subgroup C and group L. Also, a high statistically significant difference in the mean percentages of necrosis was detected among all studied groups and subgroups as revealed by ANOVA test results. Post-hoc multiple comparison test results showed a high statistically significant difference on comparing the mean percentage of necrosis in subgroup C with those of subgroups LP1, P2, LP2 and Cis (*p-values* = 0.022, 0.001, 0.000 and 0.000 respectively), and statistically non-significant difference with all other studied groups and subgroups. On comparison of the mean percentage of necrosis of subgroup P1 with that of subgroup LP1 and the mean percentage of necrosis of subgroup P2 with that of subgroup LP2, a statistically non-significant difference was detected *(p-values* = 0.996 and 0.665 respectively).

Similar to apoptosis, the results revealed an increase in the mean percentages of necrosis in subgroups treated with IC50 concentration (A2, P2, LP2) when compared to their corresponding subgroup treated with half IC50 concentration (A1, P1, LP1). The results of the unpaired t-test revealed high statistically significant differences between the mean percentage of necrosis of subgroup C and subgroup Cis as well as subgroup LP1 and LP2 and a statistically non-significant difference between the mean percentages of necrosis of subgroup A1 and subgroup A2 as well as subgroup P1 and P2.

Images of all studied groups and subgroups showing early apoptotic, late apoptotic, as well as necrotic HNO-97 cells are shown in (Figure 5).

Different superscripts in the same row mean significant difference between groups.

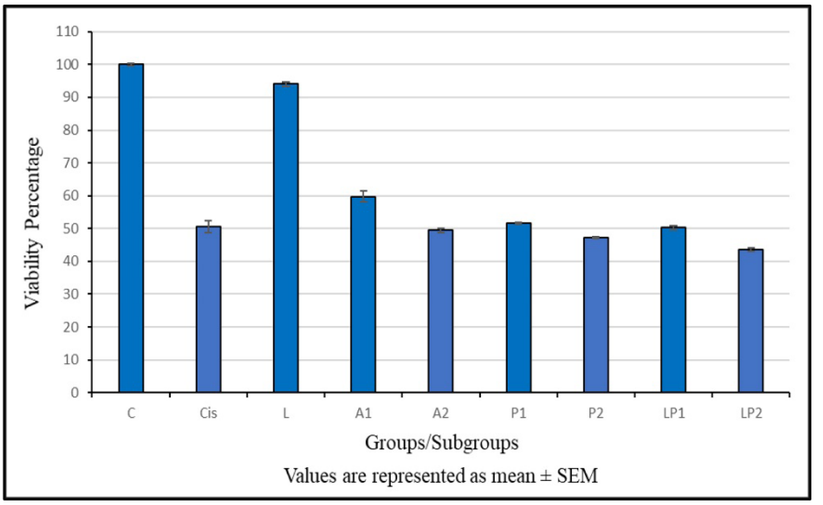

Fig. 1: A bar chart representing the mean viability percentages of all studied groups and subgroups.

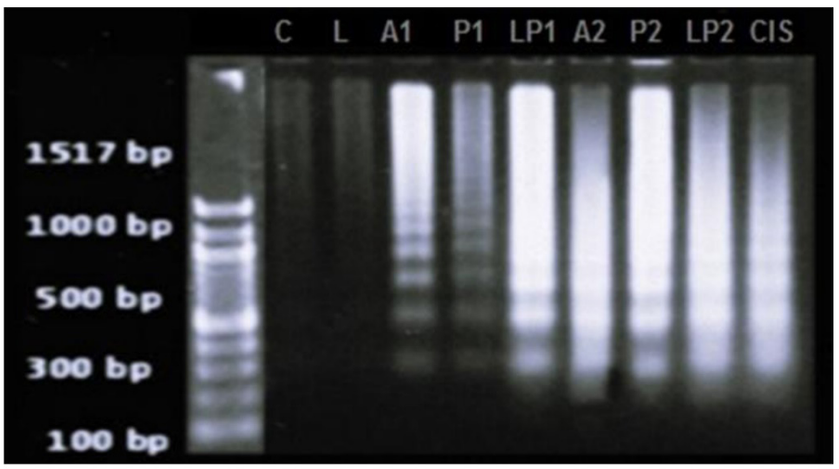

Fig. 2: Photograph showing DNA fragmentation by agarose gel electrophoresis for HNO-97 cells receiving different treatments in comparison to control DNA marker of known fragments length (leftmost lane).

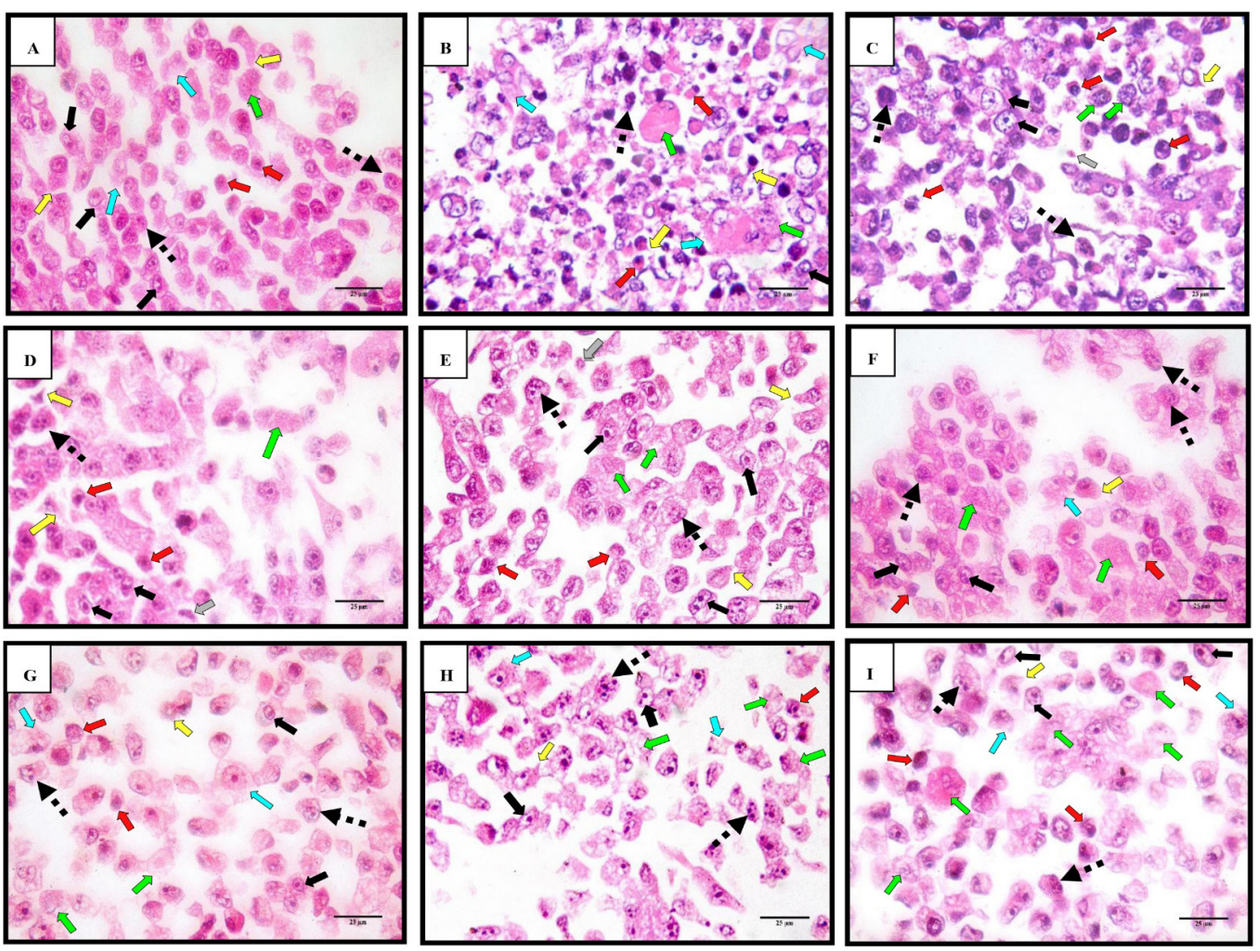

Fig. 3: Photomicrographs of all studied groups and subgroups (A: Subgroup C, B: subgroup Cis, C: group L, D: subgroup A1, E: subgroup A2, F: subgroup P1, G: subgroup P2, H: subgroup LP1, I: subgroup LP2) showing HNO-97 cells exhibiting signs of apoptosis such as shrinkage in size with pyknotic nuclei (red arrows), peripheral condensation of chromatin (black arrows), apoptotic bodies (grey arrows), membrane blebbing (yellow arrows) and nuclear fragmentation (segmented black arrows). Necrotic cells were also detected manifesting as cellular swelling along with karyolysis or karyorhexis (green arrows) and loss of membrane integrity (blue arrows). An increase in number of apoptotic and necrotic cells could be detected in subgroup Cis when compared to subgroup C as well as in subgroups treated with IC50 concentration of 5-ALA (A2, P2, LP2) when compared to subgroups treated with half IC50 concentration of 5-ALA (A1, P1, LP1) (H&E, original magnification x100 oil).

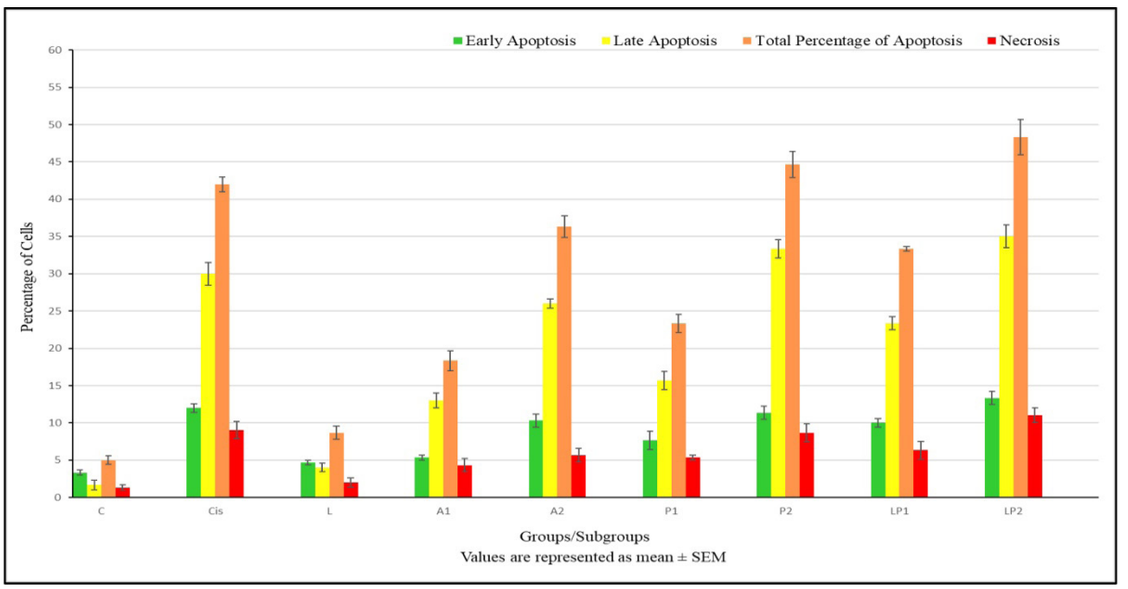

Fig. 4: A bar chart representing the mean percentages of early apoptosis, late apoptosis, total apoptosis and necrosis of all studied groups and subgroups.

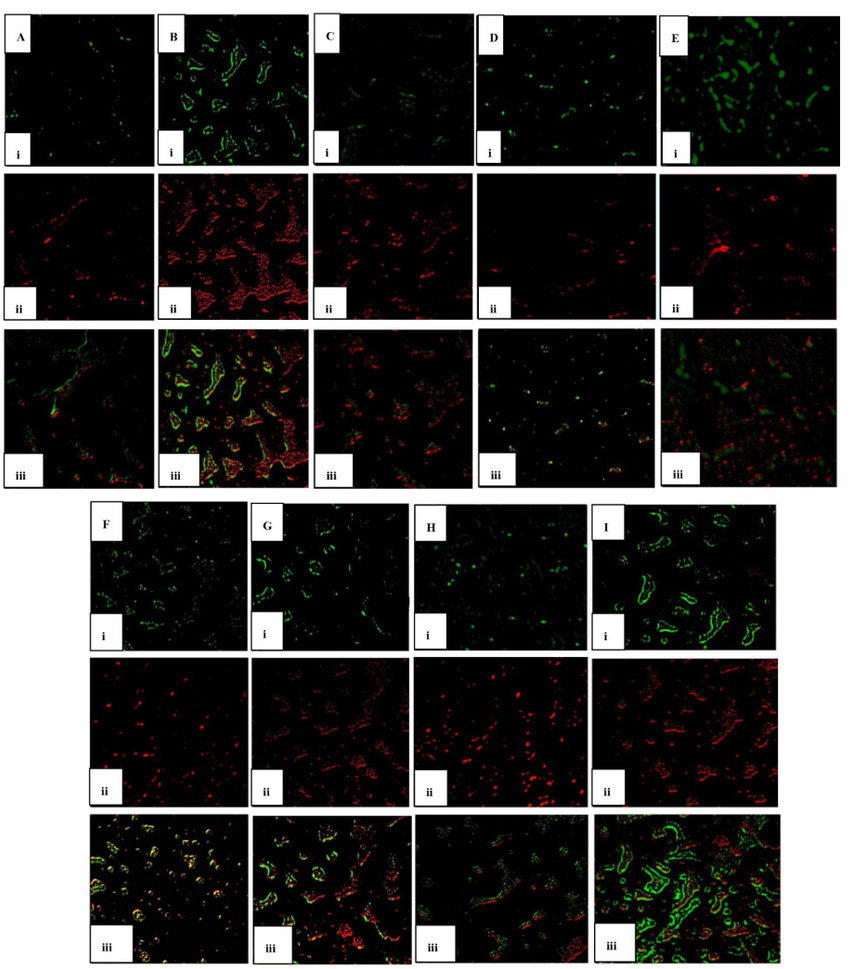

Fig. 5: Photomicrographs of all studied groups and subgroups (A: Subgroup C, B: subgroup Cis, C: group L, D: subgroup A1, E: subgroup A2, F: subgroup P1, G: subgroup P2, H: subgroup LP1, I: subgroup LP2) showing i) Ann-V+ cells ii) PI+ cells and iii) Ann-V/PI+ cells (Ann-V/PI, original magnification x40).

Table 2: Statistical results of mean viability percentages of all studied groups and subgroups

		C	Cis	L	A1	A2	P1	P2	LP1	LP2	ANOVA *p-value*
Viability Percentage	Mean ± SEM	99.99[a] ± 0.41	50.59[d] ± 1.84	94.04[b] ± 0.63	59.76[c] ± 1.72	49.52[d] ± 0.63	51.69[d] ± 0.23	47.23[de] ± 0.33	50.23[d] ± 0.63	43.59[e] ± 0.43	0.000*
	Unpaired T-test *p-value*	0.001*			0.005*		< 0.001*		0.001*		

* Significance at *p-value* < 0.05 level.

Table 3: Statistical results of mean percentages of early apoptosis, late apoptosis, total apoptosis and necrosis of all studied groups and subgroups

		C	Cis	L	A1	A2	P1	P2	LP1	LP2	ANOVA *p-value*
Percentage of Early Apoptosis	Mean ± SEM	3.33[a] ± 0.33	12.00[d] ± 0.58	4.67[a,b] ± 0.33	5.33[a,b] ± 0.33	10.33[c,d] ± 0.88	7.67[b,c] ± 1.20	11.33[c,d] ± 0.88	10.00[c,d] ± 0.58	13.33[d] ± 0.88	0.000*
	Unpaired T-test *p-value*	<0.001*			0.006		0.070		0.034*		
Percentage of Late Apoptosis	Mean ± SEM	1.67[a] ± 0.67	30.00[d,e] ± 1.53	4.00[a] ± 0.58	13.00[b] ± 1.00	26.00[c,e] ± 0.58	15.67[b] ± 1.20	33.33[d] ± 1.20	23.33[c] ± 0.88	35.00[d] ± 1.53	0.000*
	Unpaired T-test *p-value*	<0.001*			<0.001*		<0.001*		0.003*		
Percentage of Total Apoptosis	Mean ± SEM	5.00[a] ± 0.58	42.00[d,e] ± 1.00	8.67[a] ± 0.88	18.33[b] ± 1.33	36.33[c,e] ± 1.45	23.33[b] ± 1.20	44.67[d] ± 1.76	33.33[c] ± 0.33	48.33[d] ± 2.40	0.000*
	Unpaired T-test *p-value*	<0.001*			0.001*		0.001*		0.003*		
Percentage of Necrosis	Mean ± SEM	1.33[a] ± 0.33	9.00[d,e] ± 1.15	2.00[a,b] ± 0.58	4.33[a,b,c] ± 0.88	5.67[a,b,c,d] ± 0.88	5.33[a,b,c,d] ± 0.33	8.67[c,d,e] ± 1.20	6.33[b,c,d] ± 1.20	11.00[e] ± 1.00	0.000*
	Unpaired T-test *p-value*	0.003*			0.345		0.056		0.041*		

* Significance at *p-value* < 0.05 level.

DISCUSSION

5-aminolevulinic acid-mediated photodynamic therapy is an emerging and promising area of research for cancer treatment as it is considered a minimally invasive therapeutic technique that specifically targets neoplastic cells while sparing normal cells and thus has fewer side effects than conventional treatment options[18].

In the present study, the MTT cytotoxicity assay results demonstrated that LLLT only (650 nm wavelength and 10 J/cm^2 fluence) had obvious cytotoxicity on HNO-97 cells where the mean viability percentage of this group decreased significantly on comparing it with that of untreated control cells. This indicates that the used laser parameters in the 5-ALA-PDT protocol were safe, as they did not promote the proliferation of cancer cells. These findings align with previous studies performed by Murayama *et al*. (2012) and Schalch *et al*. (2019) who reported that LLLT significantly hindered the cell viability of the tested cancer cell lines (human-derived glioblastoma A-172 cells and HNC SCC9 cells respectively). Murayama *et al*. (2012) justified this cytotoxic effect by the cell cycle arrest in the G0 phase as a result of LLL irradiation, whereas Schalch *et al*. (2019) explained this by the reduction of the mitochondrial activity and enhancement of apoptosis by improving caspase 3 activity[19,20]. The reduction in mean viability percentage due to LLL irradiation in the current study might be attributed to the diminution of nutrition in the culture medium surrounding the cells, which results in hindering cell viability and boosting apoptosis rate. This explanation finds support in a study by Zhou *et al*. (2022) where they noted similar effects on nucleus pulposus (NP) cells resulting from nutrition depletion[21].

In addition, MTT assay results revealed an obvious cytotoxic effect of 5-ALA on HNO-97 where its IC50 was equal to 1.35 mM. With regard to the 5-ALA only treatment subgroups, the mean viability percentages decreased significantly when compared to untreated cells. This outcome was in accordance with the research outcomes of Fahmy and Fahmy (2020), Jalili-Nik *et al*. (2021) and

Kumar *et al*. (2023) who reported a cytotoxic effect of 5-ALA on bladder T24 cancer cells, glioblastoma U87MG cells and three different hepatocellular carcinoma (HuH7, Hep3B, and HepG2) cell lines respectively[13,22,23]. Jalili-Nik *et al*. (2021) justified this finding by inducing cell cycle arrest and suppression of cyclin D1 expression[23].

In contrast to our results regarding the LLLT group and 5-ALA alone groups, Abo-Zeid *et al*. (2018), Ma *et al*. (2020) and Shi *et al*. (2021) observed that separate applications of 5-ALA or laser which were utilized in the 5-ALA-PDT did not trigger cytotoxicity to any of the various cell lines tested in their experiments including oral squamous cell carcinoma SCC25 cells[24-26].

Our results regarding the 5-ALA-PDT group were in line with the well-established hypothesis of 5-ALA-PDT having a cytotoxic effect on different cancer cell lines, where the mean viability percentages of HNO-97 cells treated with 5-ALA-PDT significantly declined. Consistent with our outcome, Cornelius *et al*. (2017), Wang *et al*. (2020), Wang *et al*. (2021), Lv *et al*. (2023) and Pedrosa *et al*. (2023) indicated that 5-ALA-PDT utilizing different concentrations of 5-ALA significantly impeded the viability of various cell lines in a dose-dependent manner, due to piling up of PpIX in these cells and boosting of ROS production which ends up in the damage of mitochondria[14,27-30]. In contrast to these findings on 5-ALA-PDT efficacy, Rosin *et al*. (2018) noted that oral SCC-9 cells exhibited resistance to repeated applications of 5-ALA-PDT and this was attributed to repressed expression of PpIX, high mitochondrial density, enhanced expression of NFkB, survivin and iNOS anti-apoptotic proteins as well as activation of the PI3K/Akt/mTOR pathway which leads to increased neoplastic cell proliferation and hindered apoptosis[31].

The discrepancy between the aforementioned results regarding the LLLT, 5-ALA and 5-ALA-PDT groups can be attributed to the differences in the cell lines used for experimentation as neoplasms are inherently heterogeneous and no cell line can serve as a universal reference for all cancers originating from the same tissue. Therefore, it is not surprising that a drug or therapeutic approach may have different effects on various related cell lines[32].

Additionally, the MTT results of the current study revealed that the combined use of LLLT and 5-ALA-PDT on HNO-97 cells improved the cytotoxic effect of the 5-ALA-PDT, however, this enhancement was not statistically significant. This finding was consistent with Tsai *et al*. (2015), Negri *et al*. (2019) and de Faria *et al*. (2021) study outcomes as their studies performed on various cell lines concluded that pre-radiation with LLLT before PDT which was mediated by different photosensitizers enhanced the cytotoxic effect of PDT through boosting the cellular uptake of PS and thus enhancing the generation of ROS and ATP which are crucial for PDT-mediated apoptosis but the significance of the outcome varied depending on the type of the cell line tested[33-35].

Cisplatin was utilized in the current study as a positive control subgroup owing to its undebatable cytotoxic effect on cancer cells. MTT results revealed that the IC50 of Cisplatin on HNO-97 was 177µM. It had an obvious cytotoxic effect as the mean viability of its subgroup significantly declined in comparison to untreated cells. This outcome was consistent with previous studies performed by Mortensen *et al*. (2020), Sudo *et al*. (2020), Lv *et al*. (2023) and Omidi *et al*. (2023) who reported the cytotoxic effect of cisplatin on different cell lines in a dose and time-dependent manner[14,36-38].

The DNA fragmentation results demonstrated that neither the HNO-97 cells of the untreated negative control subgroup nor those of the LLLT group resulted in any DNA laddering, whereas DNA laddering was conspicuously observed in all other studied subgroups but in a variable manner. These results were consistent with previous research studies of Ji *et al*. (2010) and Alizadehnohi *et al*. (2012), who reported obvious DNA laddering upon incubating different cell lines utilized in their studies with similar treatments[39,40]. Ji *et al*. (2010) noticed ultimate DNA laddering 6 hours after exposing rat pheochromocytoma PC12 cell line to 5-ALA-PDT treatment protocol[39]. Also, Alizadehnohi *et al*. (2012) detected obvious DNA laddering on treating ovarian cancer A2780cp cells with cisplatin for 24 hours[40]. The slight smearing pattern of the DNA ladder in the present study denotes that some early apoptotic cells progressed into the late apoptosis stage, a finding that was further confirmed by the Ann-V/PI double staining results, or changed into secondary necrotic cells that were not efficiently eliminated by phagocytes that are lacking in in-*vitro* studies.

In further validation of the previously mentioned results of the MTT cytotoxicity assay and DNA fragmentation, the cytologic assessment results demonstrated an increase in the number of apoptotic cells as well as necrotic cells among all treatment groups with the highest number detected in the combination treatment group as well as cisplatin subgroup and this increase was also corresponding to the increase in the 5-ALA concentration. These findings were in accordance with a group of previous research studies that demonstrated the presence of apoptotic as well as necrotic cells in response to similar treatments applied in the current study[29,35,41,42]. For instance, Wang *et al*. (2021) demonstrated apoptotic OVCAR3 cells post 5-ALA-PDT and that the morphologic features of apoptosis increased in a time-dependent manner[29]. With regards to the combination treatment group, a similar finding was reported by de Faria *et al*. (2021) who noted an exaggeration in the cell death morphologic signs in the combination treatment group on its comparison with the corresponding PDT group[35]. Additionally, our cytologic assessment results regarding the cisplatin subgroup were consistent with Heymann *et al*. (2016) and Wang *et al*. (2016) who reported the appearance of morphologic alterations representing apoptosis such as decreased cell number, detachment from the neighboring cells as well as cells shrinkage of the tested cell lines post-incubation with various concentrations of cisplatin[41,42].

Regarding the Ann-V/PI double staining results, the results revealed a non-significant impact on mean percentages of apoptosis and necrosis of LLLT on HNO-97 in relation to untreated cells. This finding was supported by previous research performed by Ma *et al*. 2020, who indicated that separate application of LLLT on SCC25 had no significant impact on the apoptotic and necrotic rates[25]. About the 5-ALA only treatment group, a significant increase in the mean percentage of apoptosis was detected in the two subgroups, whereas the increase in the mean percentage of necrosis was non-significant when correlated with the mean percentages of apoptosis and necrosis of the untreated cells. This result is consistent with previous studies which indicated that separate incubation of 5-ALA with different cell lines significantly elevated the total rate of apoptosis whereas the impact on the rate of necrosis was insignificant[22,23].

Concerning the 5-ALA-PDT group, the results indicated that the mean percentage of apoptosis significantly increased in both subgroups whereas the mean percentage of necrosis increased non-significantly in the subgroup treated with half IC50 concentration of 5-ALA (subgroup P1) and significantly in the subgroup treated with IC50 concentration (subgroup P2) when compared to untreated cells. This finding was in line with previous studies performed by Wang *et al*. (2020), Ma *et al*. (2020) and Wang *et al*. (2021) who indicated that 5-ALA-PDT significantly enhanced the rate of apoptosis and necrosis of cancer cells in a dose-dependent manner but with unequal sensitivities that varied with the different cell lines utilized[25,28,29]. Wang *et al*. (2021) explained the increased rate of apoptosis by the reinforced expression of Bax protein as well as the attenuated expression of Bcl-2 and caspase-3 proteins[29]. It is well established that PDT-mediated apoptosis is controlled by cytochrome c liberation, suppression of NF-kB as well as impairment of Bcl-2 due to laser irradiation. Furthermore, the increase in the mean percentage of necrosis in subgroup P2 was in line with Kumar *et al*. (2023) who reported that 5-ALA-PDT triggered p53-dependent necrotic cell death of hepatocellular carcinoma (HuH7) cells[13]. The justification for this outcome is that 5-ALA-PDT-mediated necrosis is usually associated with increased doses of 5-ALA-PDT and perfusion of PpIX to cell cytoplasm components other than the mitochondria[43,44].

Referring to the combination group of LLLT and 5-ALA-PDT, the results showed that pre-radiation of HNO-97 enhanced the killing effect of 5-ALA-PDT as the mean percentage of apoptosis increased significantly and the mean percentage of necrosis increased in a non-significant manner in the subgroup treated with half IC50 concentration of 5-ALA (subgroup LP1) when compared to the corresponding 5-ALA-PDT subgroup treated with the same concentration of 5-ALA (subgroup P1). For the subgroup treated with IC50 concentration of 5-ALA (subgroup LP2), both the mean percentages of apoptosis and necrosis non-significantly increased in comparison to the corresponding 5-ALA-PDT subgroup (subgroup P2). A similar finding was reported by Tsai *et al*. (2015) and Negri *et al*. (2019) who indicated that pre-radiating neoplastic cells before PDT-mediated by various PSs enhanced the apoptotic rate owing to the reinforced uptake of the PS and increased ATP generation[33,34]. As for the mild increase in the mean percentage of necrosis in the combination treatment group of LLLT and 5-ALA-PDT, it may be attributed to the slight thermal effect of the laser irradiation since this treatment group was irradiated twice as it is well established that increased laser dosages end up in necrosis[45].

The mean percentages of apoptosis and necrosis of the positive control cisplatin subgroup significantly increased in comparison to the untreated cells; a finding that was previously supported by Alizadehnohi *et al*. (2012), Wang *et al*. (2016) and Mortensen *et al*. (2020) who concluded in their studies that incubation of the various cell lines employed in their studies with various doses of cisplatin resulted in the boosting of the apoptosis rate as well as a mild increase in the necrosis rate[36,40,42].

CONCLUSION

Considering the results of the current study, 5-ALA-PDT exhibited encouraging outcomes as a potential therapeutic modality for tongue squamous cell carcinoma since it showed an obvious cytotoxic effect on HNO-97 cell line primarily by enhancing apoptosis in a dose-dependent manner. Additionally, separate applications of LLL irradiation and 5-ALA had a cytotoxic effect on HNO-97 cells. Moreover, the combination of LLLT and 5-ALA-PDT showed promising results in reinforcing the cytotoxic effect of 5-ALA-PDT predominantly by intensifying the rate of apoptosis.

RECOMMENDATIONS

In an attempt to enhance the cytotoxic effect of 5-ALA-PDT and its combination with LLLT, it is highly recommended to utilize different laser parameters such as altering the wavelength, power, irradiation time and spot size. Additionally, it is recommended to carry on similar studies on normal mucosal cell lines as well as on animal models to further validate the safety of employing 5-ALA-PDT and its combination with LLLT as a therapeutic modality for tongue squamous cell carcinoma.

ETHICS APPROVAL

The research protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Dentistry, Ain Shams University, approval number: FDASU-Rec EM012102. All procedures were performed in compliance with relevant instructions, guidelines and regulations.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير السام التآزري للإشعاع بالليزر منخفض المستوى و العلاج بالليزر الضوئي الديناميكي القائم على حمض ال ٥-أمينوليفيولينيك على خط خلايا سرطان اللسان الحرشفي

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المقدمة: سرطان الخلايا الحرشفية في اللسان هو نوع شائع من سرطان الفم ويزداد حدوثه بشكل كبير في الأونة الأخيرة. العلاجات التقليدية لها نجاح محدود نظرا لشدة خطورة أثارها الجانبية لذلك يركز الباحثون حاليا على اختراع علاجات أقل توغلًا حيث تستهدف الخلايا السرطانية على وجه التحديد.

أحد العلاجات الواعدة هو العلاج بالليزر الضوئي القائم على حمض ال ٥ – أمينوليفولينيك ، والذي يقوم على تنشيط محسس ضوئيًا داخل الخلايا السرطانية بشكل انتقائي عن طريق مصدر ضوئي مما يؤدي الى تراكم مركبات الأكسجين التفاعلية الضارة و بالتالي موت الخلايا السرطانية دون الإضرار بالخلايا الطبيعية.

الهدف من البحث: تقوم الدراسة بتقييم فعالية العلاج بالليزر الضوئي القائم على حمض ال ٥ – أمينوليفولينيك و تقييم التأثير الفردي للإشعاع بالليزر منخفض المستوى بالإضافة إلى تأثير العلاج المجتمع بالليزر الضوئي القائم على حمض ال ٥ – أمينوليفولينيك والإشعاع بالليزر منخفض المستوى على خط خلايا سرطان اللسان الحرشفيHNO-٩٧.

المواد و طرق البحث: اشتمل التقييم على قياس نسبة بقاء الخلية ، والكشف عن موت الخلايا المبرمج من خلال تجزئة الحمض النووي ، ومراقبة التغيرات في مورفولوجيا الخلايا ، واستخدام صبغة تسمى/ Annexin-V Iodide Propidium للتمييز بين أنواع موت الخلايا المختلف الناجم عن العلاجات المطبقة في هذه الدراسة.

النتائج: أظهرت النتائج أن العلاج بالليزر الضوئي القائم على حمض ال ٥ – أمينوليفولينيك والإشعاع بالليزر منخفض المستوى، سواء على حدى أو مجتمعين، كان لهما تأثير سام ملحوظ على خلايا سرطان اللسان الحرشفي ، حيث قللت من قدرة الخلية على البقاء بشكل يعتمد على الجرعة. أدت العلاجات المطبقة في هذه الدراسة الى موت الخلايا المبرمج حيث ظهرت زيادة ملحوظة في عدد موت الخلايا المبرمج المبكر والمتأخر. أيضا، أظهر العلاج المجتمع للإشعاع بالليزر منخفض المستوى وحمض ال ٥ - أمينوليفولينيك أكثر تأثيرا بشكل ملحوظ في زيادة موت الخلايا المبرمج.

الاستنتاجات: و بناءا عليه، استنتجت الدراسة أن العلاج بالليزر الضوئي القائم على حمض ال ٥ – أمينوليفولينيك فعال في قتل خلايا سرطان اللسان الحرشفي وأن الإشعاع المسبق للخلايا يعزز قتل الخلايا عن طريق موت الخلايا المبرمج.