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In vitro comparative effects of laser photodynamic therapy with methylene blue or aminolevulinic acid on oral squamous cell carcinoma cell line

Maryam Khosravi[1], Amir Mansour Shirani[1], Maryam Ostadsharif[2], Ramin Saravani[3]

[1]Department of Oral Medicine, School of Dentistry, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran, [2]Department of Medical Basic Sciences, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran, [3]Cellular and Molecular Research Center, Research Institute of Cellular and Molecular Sciences in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran

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Address for correspondence:
Dr. Amir Mansour Shirani, Department of Oral Medicine, School of Dentistry, Isfahan Branch, Islamic Azad University, Isfahan, Iran.
E-mail: am_shirani@khuisf.ac.ir

ABSTRACT

Background: This study aimed to compare the effects of laser photodynamic therapy (PDT) with methylene blue (MB) or aminolevulinic acid (ALA) on the oral squamous cell carcinoma (OSCC) cell line.

Materials and Methods: In this *in vitro* experimental study, the C152 (KB) OSCC cell line was cultured in a culture medium containing 10% fetal bovine serum. The cells were exposed to 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM concentrations of MB and ALA alone and combined with diode laser irradiation with 660 nm wavelength, 40 mW power, and 10 J/cm^2 energy density in continuous-wave mode perpendicular to the surface. Cell viability was assessed using the methyl thiazolyl tetrazolium assay and compared among the groups by the Kruskal–Wallis test.

Results: The results showed that the reduction in cell viability in the MB + laser and ALA + laser groups was greater than that in the MB and ALA groups without laser ($P < 0.001$). Significant differences were noted in cell viability in the presence of some different concentrations of MB and ALA ($P < 0.05$), such that by an increase in their concentration, cell viability decreased. Cell viability in the MB + laser group was significantly lower than that in the ALA + laser group in some photosensitizer concentrations ($P < 0.05$).

Conclusion: Within the limitations of this *in vitro* study, the results showed that laser PDT with MB (high concentrations) was more effective than laser PDT with ALA against the OSCC cell line.

Key Words: Aminolevulinic acid, carcinoma, methylene blue, photochemotherapy, squamous cell

INTRODUCTION

Cancer is a common cause of morbidity and mortality worldwide. Despite the significant advances made in cancer prevention, diagnosis, and treatment, a definite cure for cancer has not been identified yet, and it is still a common health dilemma worldwide. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the maxillofacial region. It can cause severe disfiguration and adversely affect patients' quality of life. It has been documented that tobacco use, high

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alcohol consumption, viral infections, malnutrition, chronic stimulation, poor oral hygiene, low intake of fruits and vegetables, and genetics are risk factors for it. Several treatment options such as surgical management, radiotherapy, and chemotherapy have been proposed for it with different successes and complications.[1-5]

Photodynamic therapy (PDT) is a noninvasive treatment modality that involves the use of a dye as a photosensitizer (PS) and a suitable wavelength of light source that activates the PS. The PS binds to target cells, and it is activated by photons of the light source.[6] Tumor destruction from PDT can occur by both programmed (apoptotic) pathways and nonprogrammed (necrosis) pathways. PS is believed to preferentially concentrate in the rapidly dividing cells of malignancy. Therefore, ideally, the PDR is lethal to tumors without affecting normal tissue.[7]

PDT was used in some studies for oral and head-and-neck cancers and premalignant lesions.[7-30] PDT may be a suitable treatment option for patients with extensive lesions, those with postoperative recurrence, patients suffering from complications of radiotherapy, those not consenting to surgery, and immunocompromised patients.[16] PDT for the management of skin cancer has advantages for esthetics.[17]

As a gap of information regarding the efficacy of PDT with aminolevulinic acid (ALA) against the OSCC cell line, this study aimed to compare the effects of laser PDT with methylene blue (MB) and ALA on the OSCC cell line. According to data searches, there was no similar study up to now.

MATERIALS AND METHODS

This *in vitro* experimental study was conducted on KB cancer cell line (C152) purchased from the Pasteur Institute of Iran, Tehran, in 25 cm^2 flasks (SPL, Germany). The study protocol was approved by the Ethics Committee of Islamic Azad University, Isfahan branch (IR.IAU.KHUISF.REC.1400.100). After the disinfection of flasks with 70% alcohol and observing cell density under an inverted microscope (Leica, Germany), they were incubated at 37°C with 5% CO_2 and 95% humidity. The flasks were monitored daily in terms of cell proliferation and morphology and the absence of bacterial and fungal contamination. The culture medium of the flasks was refreshed every 1–2 days. For this purpose, under a laminar good (Airflow, Jal Tajhiz, Iran), the old culture medium and cells were transferred into sterile tubes and centrifuged at 1500 rpm for 5 min. A fresh culture medium containing 10% fetal bovine serum (Gibco, USA), bicarbonate buffer, amino acids, vitamins, 100 U/mL of penicillin, and 100 U/mL of streptomycin was then added to the tubes. When the cell density in the flasks reached over 85%, the cells were transferred into sterile Falcon tubes (SPL, Germany) and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, the fresh culture medium was added to the remaining cell sediment, and the suspension was administered among new flasks.

Cell counting

For cell counting, 50 µL of the cell suspension was mixed with 50 µL of 0.1% trypan blue (Merck, Germany) in phosphate-buffered saline, and the cells were counted using a Neubauer chamber. For this purpose, a certain volume of cell suspension was collected and dispensed under the Neubauer slide. Dead cells are stained with trypan blue, whereas the viable cells do not uptake it. Viable cells present in the four outer squares of the chamber were counted, and the mean number of viable cells was calculated. The number of cells in each milliliter of cell suspension was calculated using the following formula:

Number of cells/mL = Mean number of counted cells × 10^4 × dilution coefficient.

Assessment of cytotoxicity by the methyl thiazolyl tetrazolium assay

For cytotoxicity assessment, 5 × 10^3 cells were seeded in each well of 96-well plates (SPL, Italy) and incubated for 24 h. The cells were divided into five groups:

Group 1 – different concentrations of MB alone, Group 2 – different concentrations of ALA alone, Group 3 – different concentrations of MB with laser, Group 4 – different concentrations of ALA with laser, and Group 5 – controls without PS alone with/or laser. Eight wells were assigned to different concentrations of each PS.

The cells in Group 1 were subjected to 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM concentrations of MB (Merck, Germany), whereas the cells in Group 2 were exposed to 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM concentrations of ALA (Merck, Germany). As mentioned above, the cells in Groups 3 and 4 were subjected to different

concentrations of MB and ALA, respectively, and received laser irradiation.

Laser irradiation

The diode LTR laser (Behsaz Gostar, Iran), 660 nm wavelength, 40 mW, 0 Hz, 250 s, 1 cm^2 irradiation area, 10 J/cm^2, perpendicular and near contact to the plate and at room temperature was irradiated [Figure 1].

Group 5 served as the control group. The control cells were not exposed to any PS and did not undergo laser irradiation.

The cells were then incubated for 24 h. After 24 h, the overlaying medium was removed, and the cells were rinsed with fetal bovine serum. Next, 5 µL of the methyl thiazolyl tetrazolium dye (5 mg/mL) was added to each well, and the plate was incubated at 37°C for 4 h. After 4 h, the culture medium was gently extracted from the wells by a syringe. For complete dissolution of formazan crystals in each well, 200 µL of dimethyl sulfoxide was added to each well, and then, the optical density of each well was read at 590 nm wavelength by a multimode microplate reader (BioTek, Synergy, USA), compared with the control group. The results were reported as the percentage of treated cells compared with the control cells using the following equation:

Percentage of viable cells = Mean optical density of treated cells/mean optical density of control cells × 100.

Data were analyzed using the Kruskal–Wallis test in SPSS version 26 software (IBM, Chicago, USA) and considering $\alpha = 0.05$.

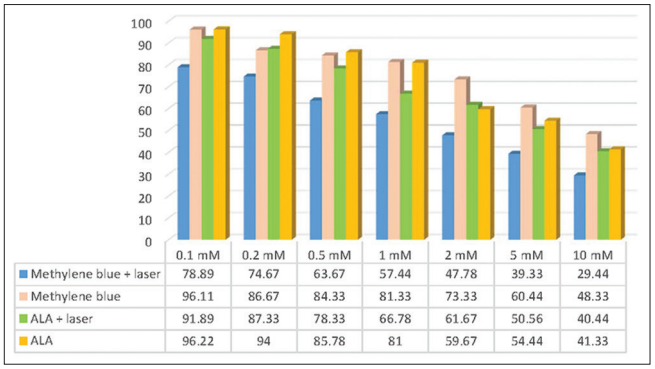
RESULTS

Table 1 presents the percentage of cell viability in the four groups.

In each group, the Kruskal–Wallis test showed a significant difference in cell viability in the presence of different concentrations of MB or ALA ($P < 0.001$). Pairwise comparisons revealed that increasing the concentration of MB or ALA markedly decreased the viability of cells ($P < 0.05$). However, the difference was no longer significant when exposed to 1 mM to 10 mM concentrations of MB or ALA ($P > 0.05$).

Figure 2 compares the four groups regarding the mean percentage of cell viability in the presence of different concentrations of MB and ALA. As shown, significant differences existed among the four groups

Figure 1: Laser irradiation of cell culture medium.



	0.1 mM	0.2 mM	0.5 mM	1 mM	2 mM	5 mM	10 mM
Methylene blue + laser	78.89	74.67	63.67	57.44	47.78	39.33	29.44
Methylene blue	96.11	86.67	84.33	81.33	73.33	60.44	48.33
ALA + laser	91.89	87.33	78.33	66.78	61.67	50.56	40.44
ALA	96.22	94	85.78	81	59.67	54.44	41.33

Figure 2: Comparison of the four groups regarding the mean percentage of cell viability in the presence of different concentrations of methylene blue and aminolevulinic acid. ALA: Aminolevulinic acid.

in 0.1 ($P < 0.001$), 0.2 ($P < 0.001$), 0.5 ($P < 0.001$), 1 ($P < 0.001$), 2 ($P < 0.001$), 5 ($P < 0.001$), and 10 mM ($P < 0.001$) concentrations of PSs.

By pairwise comparing of different concentrations of MB and ALA, it was showed that MB + laser had meaningful efficacy in reducing cell viability compared to MB alone ($P < 0.001$). ALA + laser had meaningful efficacy in reducing cell viability compared to ALA alone in 0.5 mM ($P = 0.029$) and 1 mM ($P = 0.045$). MB had meaningful efficacy in reducing cell viability compared to ALA in 0.2 mM ($P = 0.025$) and 2 mM ($P = 0.012$). MB + laser had meaningful efficacy in reducing cell viability compared to ALA + laser in 0.2 mM ($P = 0.023$) and 2 mM ($P = 0.012$).

DISCUSSION

PDT is a noninvasive modality for cancer treatment, which may be used alone or as an adjunct to surgery,

Table 1: Percentage of cell viability in four groups

Group	*n*	Concentration (mM)	Minimum	Maximum	Mean	SD
MB + laser	9	Control	100	100	100.00	0.000
	9	0.1	76	83	78.89	2.205
	9	0.2	70	80	74.67	3.162
	9	0.5	59	70	63.67	3.391
	9	1	52	62	57.44	3.206
	9	2	42	52	47.78	2.991
	9	5	32	46	39.33	4.500
	9	10	25	33	29.67	2.291
MB	9	Control	100	100	100.00	0.000
	9	0.1	95	98	96.11	1.167
	9	0.2	83	90	86.67	2.345
	9	0.5	81	88	84.33	2.550
	9	1	78	86	81.33	2.915
	9	2	70	78	73.33	2.828
	9	5	56	66	60.44	3.245
	9	10	45	52	48.33	2.693
ALA	9	Control	100	100	100.00	0.000
	9	0.1	93	99	96.22	1.856
	9	0.2	91	96	94.00	2.000
	9	0.5	81	89	85.78	2.774
	9	1	77	85	81.00	2.739
	9	2	56	64	59.67	2.398
	9	5	50	60	54.44	3.575
	9	10	34	47	41.33	4.213
ALA + laser	9	Control	100	100	100.00	0.000
	9	0.1	89	95	91.89	2.028
	9	0.2	82	91	87.33	2.915
	9	0.5	74	83	78.33	2.872
	9	1	62	72	66.78	3.632
	9	2	57	66	61.67	2.915
	9	5	47	55	50.56	3.046
	9	10	35	45	40.44	3.046

ALA: Aminolevulinic acid; SD: Standard deviation; MB: Methylene blue

chemotherapy, or radiotherapy. Following the activation of the PS, it undergoes a transition from a low-energy ground state to a higher energy state. This transition results in the formation of singlet oxygen, reactive oxygen species, and other reactive free radicals that are toxic to certain cells and microorganisms. Based on this selective cytotoxicity, PDT has been utilized in medicine for the treatment of a variety of conditions such as various cancers or microorganisms (photoactivated disinfection).[6] It has shown promising results in many premalignant conditions such as oral lichen planus and carcinoma *in situ*.[18-21] At now, the efficacy of some PSs with different laser wavelengths on the death of malignant OSCC cells is not clear, thus this study was done to compare two PSs MB and ALA alone or with 660 nm laser on the death of malignant oral SCC cells.

In the present study, MB was used as a PS due to its application for oral SCC.[21,22] ALA was also used in this study which is a nonporphyrin PS highly selective for tumoral cells.[25]

The present results showed a significant difference in cell viability when treated with different concentrations of MB with or without laser. Minimum cell viability was recorded in a 10 mM concentration of MB. Furthermore, the reduction in cell viability was more significant in the MB + laser group than in MB alone. Like the present findings, Vahidi Banehkohal *et al*.[23] reported a higher rate of cell death in the presence of a 2 μg/mL concentration of MB compared with 0.5 μg/mL. In other words, increasing the concentration of MB decreased cell viability. Aghahosseini *et al*.[18] showed that PDT with MB was an effective treatment modality for oral lichen planus. It is a premalignant lesion and may transfer to SCC, but in SCC, the effects and results may be different.

The effect of ALA in this study was similar to MB as minimum viability was recorded when oral carcinoma cells were treated with 10 mM ALA. Moreover, ALA + laser was more effective than ALA alone in reducing cell viability. Jerjes *et al*.,[12] Han *et al*.,[24] Chen *et al*.,[25] and Yang *et al*.[26] showed promising results of PDT with ALA for the treatment of precancerous and OSCC lesions, which agreed with the present findings.

Comparison of the mean cell viability in the four groups in escalating concentrations of PS s revealed significant differences. In some concentrations of PS, PDT with MB was more effective than ALA. As in these concentrations, MB alone was more effective than ALA, this efficacy may be related to PS than PDT.

According to this study, PDT with 10 mM MB and 10 J/cm^2 660 nm diode laser had the best efficacy for killing vital OSCC cells, but as in different studies, different protocols were used, more studies to investigate the best treatment protocol for OSCC are necessary. *In vivo* studies also need for considering better treatment protocols for these patients.

In a 2022 study from Spain, a new PDT device was designed based on LED technology and used for four cases of premalignant and one superficial basal cell carcinoma with good results.[29] LEDs have similar and different effects compared to lasers, thus more comparisons between these two devices are necessary in this field.

In a mini systematic review in 2022 for the evaluation of light-activated phytochemicals in PDT for cancer, some questions, such as most effective PS, its administration, the time of irradiation, light source, and sensitivity of cells toward PS, were considered for more evaluation.

PDT effects can be direct with destroying the tumoral mass or indirect effects such as vascular effects, apoptosis induction, inflammation, and generation of an immune response. This study only evaluated the direct effect of PDT, thus this is a limitation of this study, and *in vivo,* studies are needed to complete the evaluation of this technique.

To the best of our knowledge, this study was the first to compare the efficacy of PDT (660 nm diode laser) with MB and ALA against OSCC, which was a strength of this study. However, no previous study is available on this topic to compare our results with. Not assessing cell viability based on the duration of exposure of cells was another limitation of this study, which should be addressed in future studies. Maybe some studies are also required to assess some aspects of this treatment in animal models. Furthermore, the efficacy of different laser wavelengths and parameters and the long-term effects of this modality should be investigated.

CONCLUSIONS

Within the limitations of this *in vitro* study, the results showed that MB is better than ALA for reducing OSCC cell viability. PDT was better than each PS alone against OSCC cells. The MB + laser group was more effective than the ALA + laser group against the OSCC cell line in some concentrations. It may be related to the better efficacy of MB than ALA.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

REFERENCES

1. Glick M, Greenberg MS, Lockhart PB, Challacombe SJ. Burket's Oral Medicine. 3th ed. USA: Wiley Blackwell; 2021. p. 211-58.
2. Neville BW, Damm DD, Allen CM, Chi AC. Oral and Maxillofacial Pathology. 4th ed. Canada: Elsevier; 2016. p. 374-89.
3. La Rosa GR, Gattuso G, Pedullà E, Rapisarda E, Nicolosi D, Salmeri M. Association of oral dysbiosis with oral cancer development. Oncol Lett 2020;19:3045-58.
4. Kirita T, Omura K. Oral Cancer Diagnosis and Therapy. Tokyo: Springer; 2015.
5. Fribley AM. Targeting Oral Cancer. USA: Springer; 2016.
6. Romanos GE. Laser Light Therapy in Dentistry Efficacy, Uses and Limitations. New York: Nova Science Publishers; 2021. p. 381-4.
7. Allison RR, Moghissi K. Photodynamic therapy (PDT): PDT mechanisms. Clin Endosc 2013;46:24-9.
8. Wang X, Li N, Meng J, Wen N. The use of topical ALA-photodynamic therapy combined with induction chemotherapy for locally advanced oral squamous cell carcinoma. Am J Otolaryngol 2021;42:103112.
9. Wang X, Jin J, Li W, Wang Q, Han Y, Liu H. Differential *in vitro* sensitivity of oral precancerous and squamous cell carcinoma cell lines to 5-aminolevulinic acid-mediated photodynamic therapy. Photodiagnosis Photodyn Ther 2020;29:101554.
10. Meulemans J, Delaere P, Vander Poorten V. Photodynamic therapy in head and neck cancer: Indications, outcomes, and

future prospects. Curr Opin Otolaryngol Head Neck Surg 2019;27:136-41.
11. Marur S, Forastiere AA. Head and neck squamous cell carcinoma: Update on epidemiology, diagnosis, and treatment. Mayo Clin Proc 2016;91:386-96.
12. Jerjes W, Upile T, Hamdoon Z, Mosse CA, Akram S, Hopper C. Photodynamic therapy outcome for oral dysplasia. Lasers Surg Med 2011;43:192-9.
13. Fan KF, Hopper C, Speight PM, Buonaccorsi G, MacRobert AJ, Bown SG. Photodynamic therapy using 5-aminolevulinic acid for premalignant and malignant lesions of the oral cavity. Cancer 1996;78:1374-83.
14. Temam S, Pape E, Janot F, Wibault P, Julieron M, Lusinchi A, *et al.* Salvage surgery after failure of very accelerated radiotherapy in advanced head-and-neck squamous cell carcinoma. Int J Radiat Oncol Biol Phys 2005;62:1078-83.
15. Rigual NR, Thankappan K, Cooper M, Sullivan MA, Dougherty T, Popat SR, *et al.* Photodynamic therapy for head and neck dysplasia and cancer. Arch Otolaryngol Head Neck Surg 2009;135:784-8.
16. Tehranchinia Z, Rahimi H, Ahadi MS, Ahadi MS. Aminolevulinic acid-photodynamic therapy of basal cell carcinoma and factors affecting the response to treatment: A clinical trial. Indian J Dermatol 2013;58:327.
17. Pei S, Kaminska EC, Tsoukas MM. Treatment of actinic keratoses: A randomized split-site approach comparison of sequential 5-fluorouracil and 5-aminolevulinic acid photodynamic therapy to 5-aminolevulinic acid photodynamic monotherapy. Dermatol Surg 2017;43:1170-5.
18. Aghahosseini F, Arbabi-Kalati F, Fashtami LA, Djavid GE, Fateh M, Beitollahi JM. Methylene blue-mediated photodynamic therapy: A possible alternative treatment for oral lichen planus. Lasers Surg Med 2006;38:33-8.
19. Henderson BW, Dougherty TJ. How does photodynamic therapy work? Photochem Photobiol 1992;55:145-57.
20. Biel MA. Photodynamic therapy and the treatment of head and neck cancers. J Clin Laser Med Surg 1996;14:239-44.
21. Andreadis D, Pavlou AM, Sotiriou E, Vrani F, Ioannides D, Kolokotronis A. Utility of photodynamic therapy for the management of oral potentially malignant disorders and oral cancer. Transl Res Oral Oncol 2016;1:1-19.
22. Le MN, Wuertz BR, Biel MA, Thompson RL, Ondrey FG. Effects of methylene blue photodynamic therapy on oral carcinoma and leukoplakia cells. Laryngoscope Investig Otolaryngol 2022;7:982-7.
23. Vahidi Banehkohal M, Tajalli H, Rahbarghazi R, Hamrahi D, Rouhi L, Vahidi Banehkohal J. The effect of laser diode and methylene blue on photodynamic therapy cells of prostate cancer at laboratory situations. J Lasers Med 2016;13:23-18.
24. Han Y, Xu S, Jin J, Wang X, Liu X, Hua H, *et al.* primary clinical evaluation of photodynamic therapy with oral leukoplakia in Chinese patients. Front Physiol 2018;9:1911.
25. Chen HM, Liu CM, Yang H, Chou HY, Chiang CP, Kuo MY. 5-aminolevulinic acid induce apoptosis via NF-κB/JNK pathway in human oral cancer Ca9-22 cells. J Oral Pathol Med 2011;40:483-9.
26. Yang DF, Lee JW, Chen HM, Hsu YC. Topical methotrexate pretreatment enhances the therapeutic effect of topical 5-aminolevulinic acid-mediated photodynamic therapy on hamster buccal pouch precancers. J Formos Med Assoc 2014;113:591-9.
27. Grant WE, Speight PM, Hopper C, Bown SG. Photodynamic therapy: An effective, but non-selective treatment for superficial cancers of the oral cavity. Int J Cancer 1997;71:937-42.
28. Triesscheijn M, Baas P, Schellens JH, Stewart FA. Photodynamic therapy in oncology. Oncologist 2006;11:1034-44.
29. Gálvez EN, Aguilera J, Fonda-Pascual P, de Gálvez MV, de Andrés-Díaz JR, Vidal-Asensi S, *et al.* Analysis and evaluation of the operational characteristics of a new photodynamic therapy device. Photodiagnosis Photodyn Ther 2022;37:102719.
30. George BP, Abrahamse H. Light-activated phytochemicals in photodynamic therapy for cancer: A mini review. Photobiomodul Photomed Laser Surg 2022;40:734-41.