The Concentration Levels of ATP in Oral Squamous Cell Carcinoma Tissues and Risk Habits Involved: An Experimental Study

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Abstract: *Introduction*: Oral cancer is a malignant neoplasm due to the carcinogenesis process. In this, tumor cells show a deregulation and reprogramming of cellular energy metabolism because production and proliferation of biomass is necessary. Adenosine 5-triphosphate (ATP) represents the currency of energy exchange, an extracellular key signaling molecule to mediate multiple cellular responses. Most metabolic energy originates from oxidation-reduction (redox) reactions in mitochondria, and this organelle could be affected by some of the known risk factors of developing oral cancer such as the intake of alcohol and tobacco. Therefore, ATP is proposed as a prognostic malignant transformation marker and predictive in the progress of cancer.

Methodology: Fifteen tissues from oral normal gingiva (controls) and fifteen from oral squamous cell carcinomas (OSCC) were collected. Risk habits were taken and recorded as presence or absence. OSCC clinical stages were taken from medical history, and these were evaluated according to AJCC. ATP was quantified with a luminescence-based (luciferin–luciferase) detection assay. Categorical variables were presented as frequencies and percentages, numerical variables as medians. The Mann-Whitney U non-parametric test was used for statistical analysis.

Results: Control group had the highest frequency of not consuming tobacco (n=11, 73.3%). OSCC groups showed a higher frequency of tumor with no invasion of close anatomic sites (<4cm) (n=9, 60%), no clear predilection between presence (n=8, 53.3%) and absence (n=7, 46.7%) of lymph node invasion and despite only one patient reported distant metastases, the more prevalent recorded clinical staging was the advanced stage (n=9, 60%). Smoking cigarettes showed significant influence ($p < 0.01$) to generate higher levels of ATP (median 1.4 µM/µL) compared to non-smokers (median= 0.5 µM/µL), contrary to the group of OSCC cases. ATP concentration was significantly ($p<0.001$) higher in the case group (median= 1.6 µM/µL) than the control group (median= 0.9 µM/µL). The results involving the aggressiveness of the disease did not show statistical difference.

Conclusions: According to this study, in non-malignant oral tissue ATP levels could be influenced by tobacco smoke but apparently this is not applied to OSCC and independently of risk habits ATP levels could be more concentrated in oral malignant tissues increasing the levels of ATP.

Keywords: Adenosine Triphosphate; Energy Metabolism; Carcinogenesis; Mouth Neoplasms; Oral Squamous Cell Carcinoma; Warburg Effect; Purinergic Signaling; Mitochondria.

INTRODUCTION

Cancer of the lip and oral cavity represented 2% of all malignant neoplasms in 2020 with 377,713 diagnosed cases and more than 177,000 deaths worldwide [1]. These tumors originate from epithelial cells and about 90% consist of oral squamous cell carcinoma (OSCC) [2]. Due to the complications associated with this tumor, OSCC constitutes a major public health challenge. It is essential to study the mechanisms involved in the establishment and progression of the OSCC.

During the carcinogenesis process, tumor cells show a deregulation and reprogramming of cellular energy metabolism [3] because production and proliferation of biomass is necessary. Since the metabolic resources within the local tissue are finite, this can lead to nutrient depletion and the accumulation of metabolic waste. The metabolites not only serve as substrates for energy and biomass generation but can also regulate gene and protein expression and influence the behavior of non-transformed cells in the vicinity of the tumor [4]. Adenosine 5'-triphosphate (ATP) represents the currency of energy exchange; it also represents a key extracellular signaling molecule that couples to specific purinergic receptors to mediate multiple cellular responses, including signaling, differentiation, proliferation and cell death [5].

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ISSN: 1929-2260 / E-ISSN: 1929-2279/26

Most metabolic energy originates from oxidation-reduction (redox) reactions in mitochondria. Some of the known risk factors of developing oral cancer, such as the intake of alcohol and tobacco could make mitochondrial functions alterations. Mitochondrial DNA is subjected to significant stress due to its proximity to the centers of production of oxygen free radicals, so that they have a varied and complex repair machinery [6]. Cigarette smoking induces oxidative stress through the excessive accumulation of reactive oxygen species (ROS) which in turn promotes apoptosis or necrosis, increases inflammatory responses and triggers the activation of several signal pathways related to cell transformation [7]. Mitochondrial dysfunction induced by cigarette smoke compounds act as blocking agents of the mitochondrial respiratory chain, causing a loss at the time of ATP generation. It has been known for decades that cigarette smoke contains many lipophilic compounds such as phenolic structures, aldehydes, and aromatic compounds that can accumulate in mitochondria and can alter the function of the mitochondrial respiratory chain, thus affecting cellular ATP production [8]. Mitochondria play an important role in alcohol metabolism through the aldehyde dehydrogenase (ALDH) enzyme; this enzyme catalyzes the conversion of acetaldehyde to acetate. When this enzyme reaches a saturation point, acetaldehyde escapes into the bloodstream and damages biomolecules such as lipids, proteins, and nucleic acids resulting in the toxic side effects associated with alcohol consumption [9]. Ethanol metabolism via alcohol dehydrogenase (ADH) generates cytosolic NADH, which is indirectly oxidized by mitochondrial electron transport depending on hydrogen transport mechanisms involving inner membrane metabolite transporters [10].

In cancer cells, the mechanisms for obtaining ATP aerobically and anaerobically with the subsequent production of lactic acid are like those in healthy tissue; however, in malignant neoplasms, the increase in ATP consumption is a consequence of the decrease in mitochondrial activity even when there is oxygen availability. This mechanism is known as aerobic glycolysis or "Warburg Effect" [11], because of the increase in lactic acid, an acidic and hypoxic microenvironment is generated that promotes tumorigenesis, invasion, metastasis and tumor survival. Hypoxia increases glucose uptake, and subsequent glycolysis reinforces the acidic and hypoxic microenvironment that is beneficial to the tumor. Acidic microenvironments are toxic to noncancerous cells; however, in acidic tumor microenvironments, the production of vascular endothelial growth factor (VEGF) is promoted, which increases angiogenesis, in addition, the immune response against tumor antigens is inhibited and the degradation of the extracellular matrix by proteases [12].

The presence of high levels of ATP, which is an important phenotypic factor of aggressive cancer cells, has been documented in various types of tumors. These cells exhibit increased cell turnover, stemness, anchorage-independence, migration, invasion, metastasis, antioxidant capacity, and drug resistance. In contrast, cancer cells with less aggressive phenotypes show low levels of ATP [13].

Hence, this present study aimed to study the expression of ATP in cancerous tissue samples of OSCC compared with oral normal tissue and analyze the effects of tobacco and alcohol intake.

MATERIALS AND METHODS

Study Subjects

Fifteen subjects who attended the Jalisco Institute of Cancerology were recruited for this experimental study, taking into consideration the oral squamous cell carcinoma sample tissues. Twenty-six subjects who attended the New Civil Hospital of Guadalajara – Juan I. Menchaca were recruited as well in consideration for the healthy oral mucosa tissue samples, making forty-one subjects total considered for this study from 2018 to 2022. The classification for the oral squamous cell carcinoma such as TNM and clinical stage was according to the 2017 eighth edition of the AJCC (American Joint Committee on Cancer) guidelines.

Ethical Approval of Studies and Informed Consent

The Ethics Committee of the Jalisco Institute of Cancerology approved the research project entitled: Prognosis in patients with squamous cell carcinoma of the oral cavity and oropharynx with number 04/15. The objective was explained to all the subjects before they participated; after they agreed on being included, the questionnaire was given and the data was collected. Written informed consent was obtained from all participants according to the 2013 Declaration of Helsinki. Similarly, an annex was included in the informed consent form for sample collection after third molar extraction, explaining the purpose of the research project, that there would be no remuneration for participation in this study, and that the study would

benefit society given the research being carried out. Tissue samples were obtained during routine procedures, such as third molar surgery

Study Groups

Control group: Subjects in the category of normal oral mucosa answered a questionnaire to collect personal data, risk habits such as the use of tobacco and alcohol consumption, as well as whether they had any disease at the time of taking the tissue sample. The only subjects taken in consideration for this study were those who attended for a third molar extraction; other patients who attended for another dental extraction or showed gingival inflammation signs were not included. After completing the questionnaire, subjects proceeded to oral clinical evaluation and consequently to the surgery of the third molar. The procedure took place under local anesthesia in the Oral and Maxillofacial department; after obtaining the tissue it was placed in a container with ice for preservation and subsequent storage in an ultra-freezer at -80°C.

Case group: Patients who presented oral squamous cell carcinoma answered a different questionnaire to collect personal data, risk habits of tobacco use and alcohol consumption, and hereditary family history of cancer of any type. Subjects with any other cancer than oral squamous cell carcinoma were not included in this study. The procedure took place in Jalisciense Institute of Cancerology under general anesthesia, in a sterile operating room environment under the Head and Neck oncology department; right after the tumor was removed, a tissue sample was obtained (approximately 1×1 cm2). The sample was disinfected in ethanol solution at 70% six times for a 30-second period each time. After that, the tissue sample was placed inside a falcon tube with PBS + penicillin/streptomycin + fungizone at 5% as an antibiotic solution, then placed into a container with ice at -4°C for transfer to the dental research institute and stored in the ultra-freezer at -80°C.

Homogenization and Measurement of ATP levels

For the homogenization of both sample tissues, the same processing was used, with the use of a lysis buffer for epithelial cells, especially since it is used for fresh or frozen animal tissues. It is based on an organic buffer, which uses a patented combination of various salts and agents that improves the extraction and stability of proteins. The homogenization process used the lysis buffer and the BEADBUG 6 Six-Position Homogenizer (115V®). The tissue sample was placed into a prefilled 2.0 mL tube with zirconium beads® with 1 mL of the buffer with the help of a micropipette of 1000 µL and then placed into the homogenizer for 10 cycles of 4350 rpm for 60 seconds each cycle with a 30-second rest interval between each cycle.

For the measurement of ATP, the Luminescent ATP Detection Assay Kit® (ab113849) was used. The luminescent ATP assay protocol involves the lysis of the previously homogenized tissue samples with the buffer and the use of the kit. 100 µL of the homogenized tissue sample aliquots were added in triplicate to a 96-well microplate. To determine the ATP concentration within these samples, the reagents of the ATP ASSAY KIT were used: 100 µL of the lysis buffer and 100 µL of the reagents. Luminescence (relative light units) was measured on a 96-well microplate luminometer®, and ATP concentration was interpolated from an ATP standard curve, and the given results are represented as micro-Molar per micro-Liter (µM/µL).

Statistical analysis: Categorical variables were presented as frequencies and percentages; numerical variables were presented as medians. The Mann-Whitney U non-parametric test was used for statistical analysis. Statistical significance was set at $p<0.05$.

RESULTS

Thirty sample tissues were collected for this study: 15 tissues from healthy oral mucosa and 15 tissues from oral squamous cell carcinoma, to measure ATP levels. The average age for the participants of the control group was 34.8±17.2 years; for the case group, an average of 59.0±12.1 years was obtained. Regarding sex distribution, the control group was predominantly female with 9 (60.0%) participants, while in the case group males presented a greater proportion with 10 (66.7%). Regarding risk habits in the control group, 11 of the participants did not present any habit (73.3%), while in the cases, tobacco usage did not present a noticeable difference, with 8 (53.3%) not using tobacco and 7 (46.7%) using it.

The TNM staging was regrouped in a dichotomous way to visualize the most aggressive groups of the disease. Taking this method into consideration, we observed the presence of tumors smaller than 4 cm more frequently (n=9, 60%). The distribution of invasion in regional lymph nodes did not show a predilection between invasive and non-invasive. Only one patient reported distant metastases, but the more prevalent clinical staging was the advanced stage (n=9, 60%) (Table **1**).

Table 1: Descriptive Characteristics of Patients with and Without OSCC (N=30)

Cases Characteristics			Controls		
Age mean	59.0 ± 12.1		34.8± 17.2		
Sex	n	%	n		%
Feminine	5	33.3	9		60.0
Masculine	10	66.7	6		40.0
Risks habits					
Tobacco use					
No	8	53.3	11		73.3
Yes	7	46.7	4		26.7
Alcohol use					
No	10	66.7	13		86.7
Yes	5	33.3	2		13.3
Disease´s characteristics					
TNM° stage					
Tumor (T)					
T0, Tis, TI and TII (<4cm)•	9	60.0		-	-
TIII and TIVA/B (>4cm)	6	40.0		-	-
Lymph nodes (N)					
Nx and N0	7	46.7		-	-
NI,N2A,B,C and N3	8	53.3		-	-
Clinical stage					
I and II	6	40.0		-	-
III and IVA/B	9	60.0		-	-

Metastasis was found in only one patient.

According to AJCC tumors shorter than 4 cm, still no had invasion to close anatomic site.

The first analysis measured ATP concentration according to risk factors to show the influence on ATP levels in each group(Figure **1** and Figure **2**) In the control group, the habit of tobacco consumption significantly influenced ($p<0.01$) the generation of higher levels of ATP in smokers with a concentration of median 1.4 µM/µL (Q1= 1.2 µM/µL, Q3= 1.7 µM/µL) compared to non-smokers (median= 0.5 µM/µL, Q1= 0.4 µM/µL, Q3= 1.1 µM/µL). On the contrary, in the case group, concentrations distributed between the presence (median= 2.1 µM/µL, Q1= 1.3 µM/µL, Q3= 2.2 µM/µL) and absence (median= 1.5 µM/µL, Q1= 1.1 µM/µL, Q3= 2.0 µM/µL) of tobacco consumption did not show significant differences. The results related to alcohol did not show significant difference in both groups (Figure **3**).

In the next analysis, a comparison of ATP concentration between the case group and the control group showed a significant difference ($p<0.001$). In the case group, the median of ATP levels was 1.6 µM/µL (Q1= 1.2 µM/µL, Q3= 2.1 µM/µL) while in the control group the median was 0.9 µM/µL (Q1= 0.5 µM/µL, Q3= 1.3 µM/µL) (Figure **4**). The results involving the aggressiveness of the disease did not show statistical difference.

DISCUSSION

ATP is an essential molecule for cell metabolism and is involved in anabolism and catabolism; it is also the fuel for muscle contraction, phosphorylation, and active transport. ATP levels reflect the positive correlation between energy demand and production. Currently there are different methods for quantifying the concentration of ATP in different populations of cells, organs and tissues of the body, among the most investigated being skeletal muscle, liver and nerve cells [14,15]. Although many tests have already been applied to the measurement of ATP in other tissues, none has been directly applied in oral mucosal tissue, much less a comparison between healthy mucosa and OSCC.

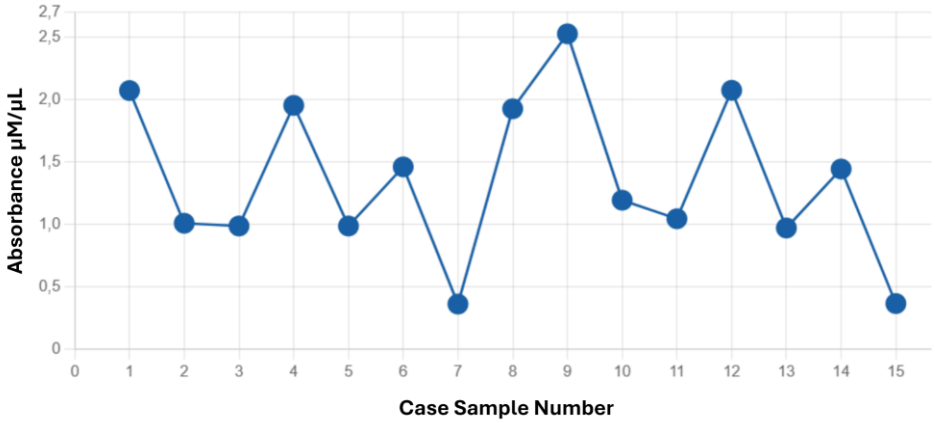

Figure 1: The figure shows the ATP levels (Absorbance µM/µL) obtained individually from a total of 15 mucosal samples from patients with oral squamous cell carcinoma (cases).

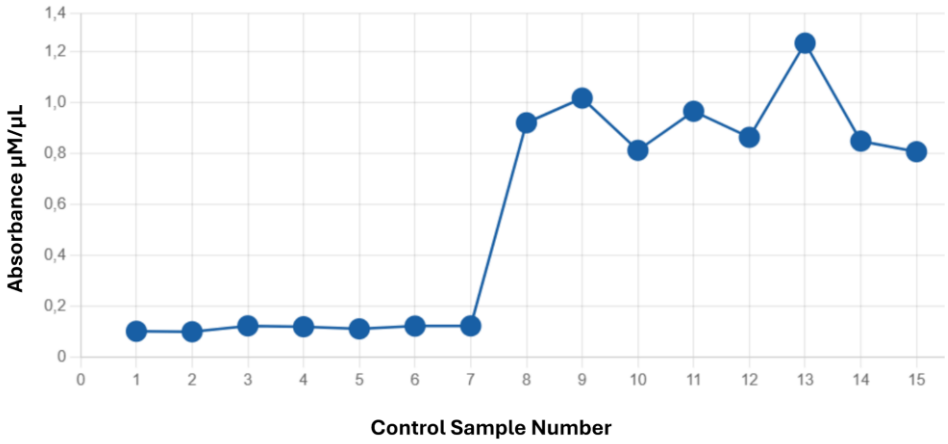

Figure 2: The figure shows the ATP levels (Absorbance µM/µL) obtained individually from a total of 15 mucosal samples collected after third molar extraction (controls).

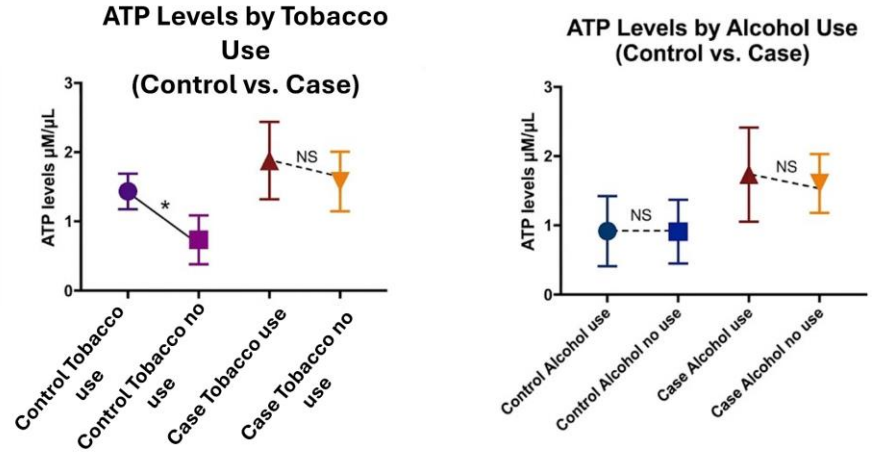

Figure 3: ATP levels µM/µL (Tabaco use/ Not use).

Control Tabaco use: Median= 1.4, Q1=1.2, Q_3= 1.7, Minimum=1.15, Maximum= 1.8.

Control Tabaco no use: Median= 0.5, Q1= 0.4, Q_3= 1.1, Minimum= 0.3, Maximum= 1.4.

Case Tabaco use: Median= 2.1, Q1= 1.3, Q_3= 2.2, Minimum=1, Maximum= 2.5.

Case Tabaco no use: Median= 1.5, $Q_{1=}$ 1.1, Q_3= 2.0, Minimum= 1.04, Maximum= 2.15.

Statistics: Man-Whitney U.

Abbreviations and symbols: ATP: Adenosin-5´-Triphosphate; NS: No significative *****p< 0.01.

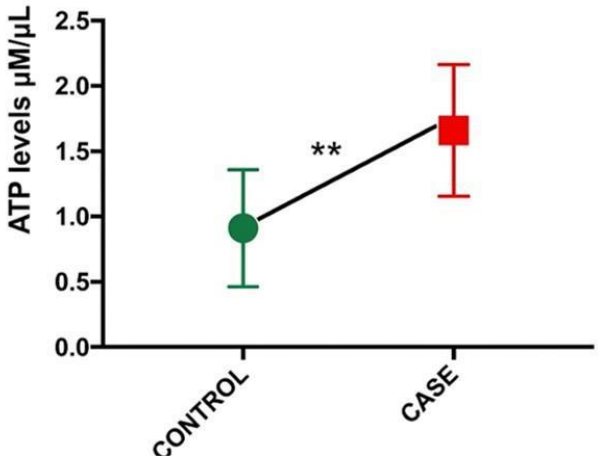

Figure 4: ATP levels µM/µL.

Control: Median= 0.9, Q1= 0.5, Q_3= 1.3, Minimum= 0.4, Maximum= 1.8;

Case: Median= 1.6, Q_1=1.2, Q_3= 2.1, Minimum= 1, Maximum= 2.6.

Statistics: Man-Whitney U.

Abbreviations and symbols: ATP: Adenosin-5´-Triphosphate; NS: No significative, **p< 0.001.

It was observed within our study that in the control group exposed to tobacco there is a greater concentration of ATP levels compared to those that do not smoke, being significant, unlike the case group. In previous studies it has been shown that cells of the healthy oral mucosa, when stimulated or exposed to risk factors such as tobacco smoke, generate harmful stimuli for the cells, because molecules composed of carbon dioxide, ammonia, carbon monoxide, hydrogen cyanide, among others increase oxidative stress and the production of free radicals which serve as promoters for damage to biomolecules such as DNA [16,17]. This increase in oxidative stress leads epithelial cells to a higher production of ATP due to the high cell division generated by the constant epithelial damage and required regeneration. Dividing cells have three main requirements: generation of ATP, high biosynthesis of macromolecules, and maintenance of the redox state [18,19].

In a tumor cell, metabolism and energy production in ATP are given by the conditions of aerobic respiration that transform glucose into pyruvate and this in turn is transformed into lactate (aerobic glycolysis), carrying out this metabolism in the cytosol [20]. Paradoxically, cancer cells that should present a greater requirement for ATP to maintain their high replication rate, even though they present a greater amount of glucose than their needs, are characterized by carrying out the transformation of glucose into lactate even in the presence of oxygen [21].

This increased glucose uptake is generated by the overexpression of glucose transporter 1 (GLUT-1) receptors in the membrane of tumor cells. It has been shown that if cells carry out respiration, their growth is less than if they carry out glycolytic metabolism, so we can interpret that the latter gives an advantage to the tumor cell because lactate serves as a substrate to produce biomolecules (catabolism) such as proteins, fatty acids and amino acids that will serve the malignant cell to increase its biomass, using both the ATP generated from cellular respiration and the ATP generated in glycolysis to stay within its microenvironment [22]. On the other hand, a group of researchers demonstrated in a squamous cell oral cancer model that fibroblasts that are part of the tumor microenvironment donate and transfer mitochondria to malignant cells [23].

This study has several limitations that should be acknowledged. First, the sample size was relatively small (15 cases and 15 controls) which limits the statistical power and the generalizability of the findings. Second, the control and case groups differed markedly in age and sex distribution, and the limited sample size precluded a robust multivariate adjustment; residual confounding therefore cannot be ruled out. Third, control tissue was obtained exclusively from gingiva (third molar extractions), whereas the OSCC samples originated from different oral subsites, so site-related differences in baseline metabolic activity may have influenced the comparison. Finally, the cross-sectional

design does not allow causal inferences regarding ATP levels and disease progression. Larger, subsite-matched, and prospective studies are therefore needed to confirm these observations.

Overall, the need for innovative and sensitive techniques to monitor ATP levels is a major challenge for therapeutic purposes due to the complexity of the pathophysiology of the disease.

CONCLUSION

According to this study, in non-malignant oral tissue, ATP levels could be influenced by tobacco smoke, but apparently this is not applicable to OSCC. Independently of risk habits, ATP levels could be more concentrated on oral malignant tissues, suggesting that elevated ATP levels may serve as a potential biomarker for malignant transformation and disease progression in oral cancer. Future studies with larger sample sizes are recommended to validate ATP as a prognostic marker in OSCC.

DECLARATION OF CONFLICTING INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Dr. Prieto-Correa: Conceptualization, methodology. Dr. Mendoza: Investigation, writing of original draft, PhD Cruz Ramos and PhD Zepeda-Olmos: formal analysis, methodology. Dr. Soto-Ávila: Data curation and Project administration. PhD López-Verdín: Conceptualization, methodology, writing — review and editing.

ACKNOWLEDGEMENTS

The corresponding authors are grateful to Dr. Carlos Perez Ortiz for his essential participation and contributions.

FUNDING

The Supporting Research Program of the Research Institute in Dentistry of the University Center of Health Sciences, University of Guadalajara supported this research to members of the national research system (PROSNI [grant number 259639/Federal fund 11482]).

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Received on 21-05-2026 Accepted on 19-06-2026 Published on 25-06-2026

https://doi.org/10.30683/1929-2279.2026.15.16