RESEARCH ARTICLE

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# The Role of Oral Microbiota in the Development of Oral Squamous Cell Carcinoma Using MicroRNA and Apoptosis-Related Gene Expression: An Exploratory Study

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## Abstract

**Background and Objective:** Oral cancer is one of the malignant tumors of the head and neck region, which is associated with high mortality rates and has various negative effects on the aesthetics of patients. Therefore, access to high-quality care for early detection and appropriate surgical and drug treatments is crucial. To this end, researchers are investigating the mechanisms of carcinogenesis in cells and identifying the factors that affect it. The aim of this study was to investigate the mechanisms by which oral microbiota contributes to carcinogenesis. **Materials and Methods:** Sixty peripheral blood samples were collected from oral squamous cell carcinoma (OSCC) patients with (30 samples) and without (30 samples) of oral infection, referred to the Cancer Institute of Tehran University of Medical Sciences. Real-time PCR was performed to determine the expression levels of *miR-92*, *miR-26, miR-486, Bak, Bax*, and *Caspase-8* genes. **Results:** *MiR-92* and *miR-26* relative expression were higher in the OSCC patients with oral infection compared to OSCC patients without oral infection. However, relative expression of *miR-486, Bak, Bax*, and *Caspase-8* was significantly decreased in patients with oral infection compared to OSCC patients without oral infection. **Conclusion:** The results showed that oral microbiome prevents apoptosis and promotes the development of cancerous tissue in OSCC patients. The identification of a link between oral infection and microRNA and apoptosis-related gene expression could provide researchers with the opportunity to formulate innovative methods for the prevention or management of OSCC.

**Keywords:** Oral squamous cell carcinoma- Oral microbiome- MicroRNA- Apoptosis-related genes

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## Introduction

Carcinogenesis is a complex process involving various signaling pathways triggered by abnormal oncogenic signals [1]. Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer globally, and it comprises a biologically diverse group of tumors [2]. Over 90% of HNSCCs occur in the mucosal surfaces of the oropharynx and larynx [3], with oral squamous cell carcinoma (OSCC) accounting for over 90% of oral cancers [4]. Oral cancers, which develop in the mouth, lip, and tongue, have a significant impact on morbidity and mortality [5], particularly in low socioeconomic status groups [6]. In the developing world, oral cancer is responsible for two-thirds of all cancer-related deaths [7]. Unfortunately, two-thirds of oral cancer cases are diagnosed at an advanced stage [8, 3].

Despite recent advances in the multidisciplinary treatment of oral cancer, patients with locally advanced disease have a poor prognosis, and disease-free patients

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are at high risk of relapse [9]. Therefore, researchers are striving to gain a better understanding of the cellular and molecular mechanisms that initiate oral tumors and facilitate metastasis [10, 11].

OSCC is the most common oral cavity malignancy, characterized by a poor prognosis and low survival rate. The etiology of this cancer is multifactorial, with tobacco and alcohol consumption being the most significant risk factors [12]. Alterations in the oral microbiome can disrupt the symbiotic relationship between microorganisms and humans, potentially leading to diseases [13]. There is increasing evidence pointing to the role of bacteria in oral cancer development. Recent studies have suggested that the oral microbiota may contribute to oral squamous cell carcinoma through the direct metabolism of carcinogens and inflammatory effects [14]. However, the association between oral microbiota and OSCC development, as well as the underlying mechanisms, are not fully understood. Therefore, it is essential to comprehend the mechanism of OSCC development to identify potential targets for molecular therapy of OSCC.

Microribonucleic acids (miRNAs) are a group of non-coding RNAs that are 19-25 nucleotides in length and play a crucial role in regulating several essential biological functions such as cell proliferation, differentiation, and apoptosis [15, 16]. Consequently, abnormal expression of miRNA may be linked to the onset and progression of cancers. Some studies indicate that the abnormal expression of miRNAs is associated with the development of OSCC [17-19].

Caspases, a family of cysteine-dependent aspartate-directed proteases, play a central role in the apoptotic cell death process [20]. The localization of caspases and the translocation of their active products are crucial for the development of the apoptotic process [21]. Studies have suggested that caspases may serve as a genetic biomarker for personal risk of various cancer types, including oral cancer [22].

The BCL-2 protein family is essential in regulating apoptotic cell death, which includes anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic proteins are *Bcl-2* and *Bcl-XL*, while the pro-apoptotic proteins consist of *Bax* and *Bak* [23]. Abnormal overexpression of pro-survival *BCL-2* family members or abnormal reduction of pro-apoptotic *BCL-2* family proteins, both resulting in the inhibition of apoptosis, are frequently found in different malignancies [24]. Given both cell proliferation and a reduced rate of apoptosis are implicated in the pathogenesis of carcinogenesis, in this study, we aim to investigate the mechanisms by which oral microbiota contributes to carcinogenesis through examining the expression of *miR-26*, *miR-92*, *miR-486, Caspase-8, Bak,* and *Bax*.

## Materials and Methods

The blood samples were collected from OSCC patients with oral infection (n = 30) and OSCC patients without oral infection (n = 30) referring to the Cancer Institute of Tehran University of Medical Sciences (ethical code: IR.SBMU.NRITLD.REC.1402.116). Patients with only primary untreated OSCC, including chemotherapy or radiotherapy, were recruited. Patients with chronic or acute inflammatory diseases were excluded.

*RNA extraction*

The blood was allowed to clot for 45 minutes to separate the serum, and then it was processed according to the instructions provided by Mircury Exiqon (Denmark). Next, the blood samples were spun at 2,500 ×g at 4°C for 15 minutes, and the resulting fluid was spun again at full speed for 15 minutes at 4°C to eliminate any remaining contaminants, including erythrocytes. The serum was stored at -70°C until it was ready to be processed for total RNA isolation. Total RNA was extracted from the serum samples using Mircury Exiqon, following the manufacturer's protocol, and miRNeasy Mini Kits and miRNeasy serum (Qiagen, Hilden, Germany) were used to extract miRs from the serum samples. The optical densities for RNA extraction were determined to be 260/280 ratio according to the manufacturer's guidelines.

*cDNA synthesis*

All cDNA samples were normalized to 2 µg, followed by microRNA cDNA synthesis using a reverse transcription system kit (Zist Royesh, Iran) and Viva 2-step RT-PCR Kit (Cat no. RTPL12) for *Bax, Bak*, and *Caspase* 8. Synthesized cDNAs from the total RNA stem-loop were used in real-time RT-PCR. Reverse transcriptase (RT) reactions contained 2 µg of RNA sample, 50 nmol/L of stem-loop RT primer, 2× RT buffer, 0.5 mmol/L of each dNTP, and 4 U/µL of M-MLV RT. The reactions (20 µL) were incubated in a PCR system at 37°C for 50 minutes and 85°C for 5 minutes, followed by holding the samples at 4°C. RNA18S was used as a housekeeping gene to normalize the expression of genes. The primer sequences of *Bak, Bax*, and *Caspase-8* are listed in Table 1.

*Real-time PCR*

Real-time PCR was carried out for miRs by SYBR Green PCR Master Mix (Zist Royesh, Iran) and for *Bak*, *Bax,* and *Caspase-8* using CinnaGreen qPCR Mix, 2X (Cat No.MM2041) in accordance with the manufacturer's instructions on a real-time PCR instrument. Each reaction was performed in a 20 µL volume, comprising 50 ng of cDNA, 2 µL of universal primer, and 10 pmol of each primer, in addition to 10 µL of 2× QuantiTect SYBR Green PCR Master Mix. The PCR amplification reaction involved denaturation at 95°C for 10 seconds, followed by 40 cycles at 62°C for 20 seconds and 72°C for 30

Table 1. Real-Time Primer Sequences

| Parameters | *Bak* | *Bax* | *Caspase 8* |
|---|---|---|---|
| F primer | ACGCTATGACTCAGAGTTCC5 | TCAGGATGCGTCCACCAAGAAG | AGAAGAGGGTCATCCTGGGAGA |
| R primer | CTTCGTACCACAAACTGGCC5 | TGTGTCCACGGCGGCAATCATC | TCAGGACTTCCTTCAAGGCTGC |

seconds. All reactions were performed in triplicate, and the comparative Ct method was used to analyze the expression level differences in each group.

*Statistical analysis*

All the data were analyzed using SPSS 20.0 Software. Differences between the groups were examined for statistical significance with an unpaired t-test. A p-value less than 0.05 was considered statistically significant.

## Results

There were no significant differences in age between the two groups. The mean standard deviation of the age for OSCC patients with oral infection and OSCC patients without oral infection were 45.2 (8.8) and 44.6 (8.1) years, respectively (p = 0.36). In 30 OSCC patients with oral infection, Porphyromonas gingivalis, Pseudomonas aeruginosa, Fusobacterium nucleatum, Streptococcus anginosus, Streptococcus mitis, Streptococcus mutans, Staphylococcus aureus Candida albicans were found. However, no evidence for the presence of oral microbiota was found in OSCC patients without oral infection.

*Expression levels of miR-92, miR-26, and miR-486*

The results revealed that the expression of *miR-92* and *miR-26* markers were detected in 86% (26 out of 30), and 93% (28 out of 30) of OSCC patients with oral infection, respectively. Statistical comparison of the positive percentage of *miR-92*, and *miR-26* markers indicated that OSCC patients with oral infection had higher expression of these markers compared with OSCC patients without oral infection (P-value < 0.001; Figure 1). Whereas the proportion of positive miR-486 significantly decreased in OSCC patients with oral infection compared with OSCC patients without oral infection (P-value < 0.001; Figure 1). Results obtained by the ΔΔCT method showed that *miR-92* and *miR-26* relative expression were 1.47 and 1.34 times higher in the OSCC patients with oral infection compared to OSCC patients without oral infection (Figure 2). However, *miR-486* relative expression was significantly decreased in patients with oral infection compared to

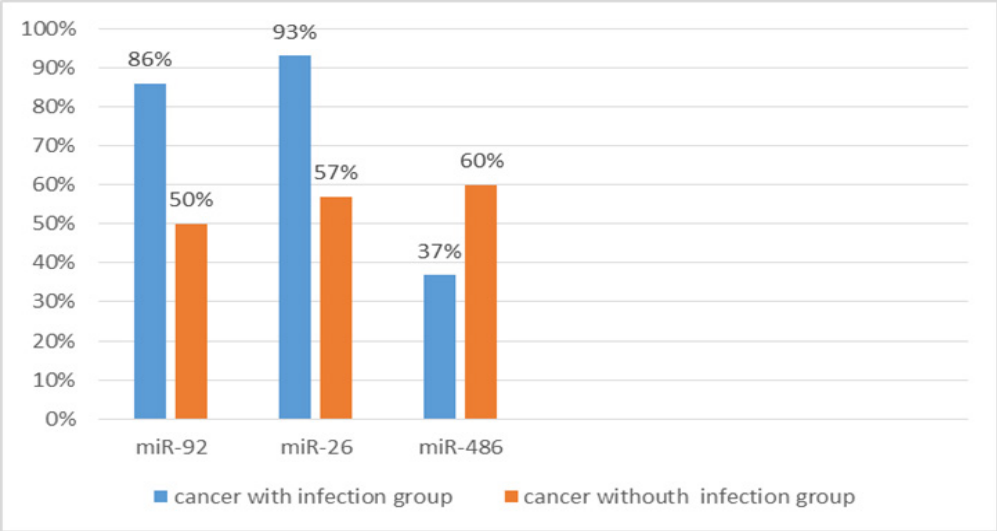


Figure 1. Percentage of Positive *miR-92, miR-26*, and *miR-486* in Patients with Oral Infection and OSCC Patients without Oral Infection.

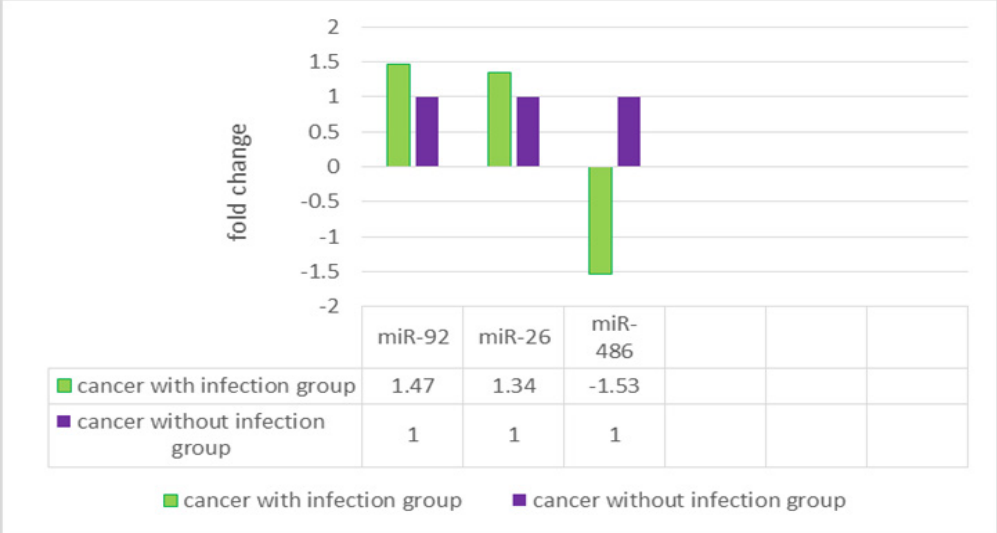


Figure 2. Expression levels of *miR-92, miR-26,* and *miR-486* in Patients with Oral Infection and OSCC Patients without Oral Infection were Measured by RT-qPCR.

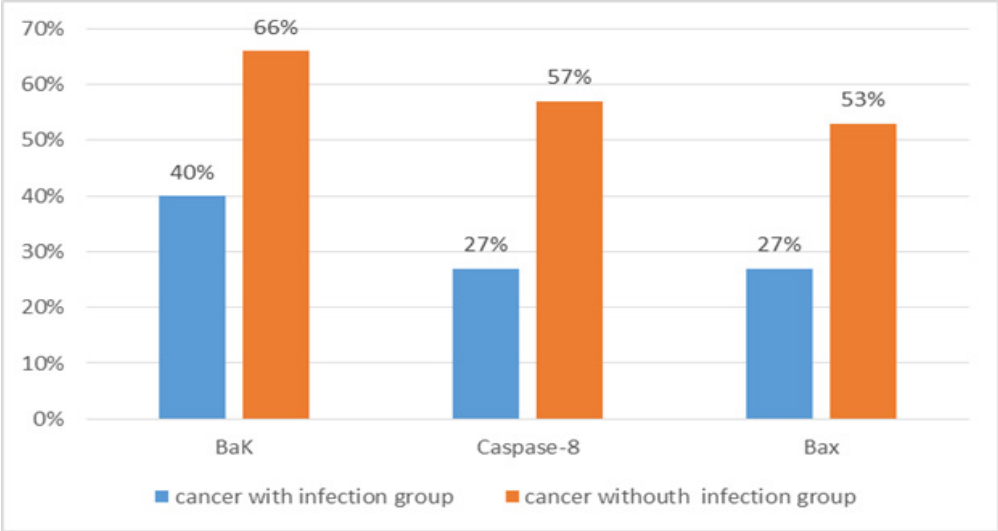


Figure 3. Percentage of Positive *Bak, Bax*, and *Caspase 8* in Patients with Oral Infection and OSCC Patients without Oral Infection

OSCC patients without oral infection (Figure 2).

*Expression levels of Bak, Bax, and Caspase 8*

The expression levels of *Bak, Bax*, and *Caspase 8* in the above cases showed the opposite trend of the expression levels. According to the findings, *Bak, Bax,* and *Caspase 8* were detected in 40% (12 out of 30) and 27% (8 out of 30) of OSCC patients with oral infection, indicating a lower expression of these markers in comparison to OSCC patients without oral infection (P-value < 0.001, as shown in Figure 3). Furthermore, the relative expression of *Bak*, *Bax*, and *Caspase 8* was significantly decreased in patients with oral infection compared to OSCC patients without oral infection (Figure 4).

## Discussion

As mounting evidence suggests that bacteria may play a role in the development of various types of cancer, it is intriguing to consider the mechanisms by which they may contribute to the carcinogenic process. In this study, we observed an increase in the expression of *miR-26* and *MiR-92*, while *miR-486, Bax, Bak,* and *Caspase-8* showed a decrease in OSCC patients with oral infections. Our data suggest that oral pathogens may contribute to tumorigenesis by decreasing the expression of proapoptotic genes and increasing the expression of antiapoptotic genes.

MicroRNAs are a type of single-stranded non-coding RNAs that are highly conserved and typically consist of 19-25 nucleotides [25]. Over the past decade, there has been significant research into the expression, functions, and mechanisms of microRNAs in cancer[26]. The overwhelming evidence suggests that microRNAs play a vital role in the critical processes of carcinogenesis and the subsequent development of cancers [26, 27]. MiRNAs not only have a role in the formation of tumors but they have also been linked to tumor suppression [28, 29]. To accurately classify specific miRNAs as oncogenes or tumor suppressors, a more in-depth analysis of their functional

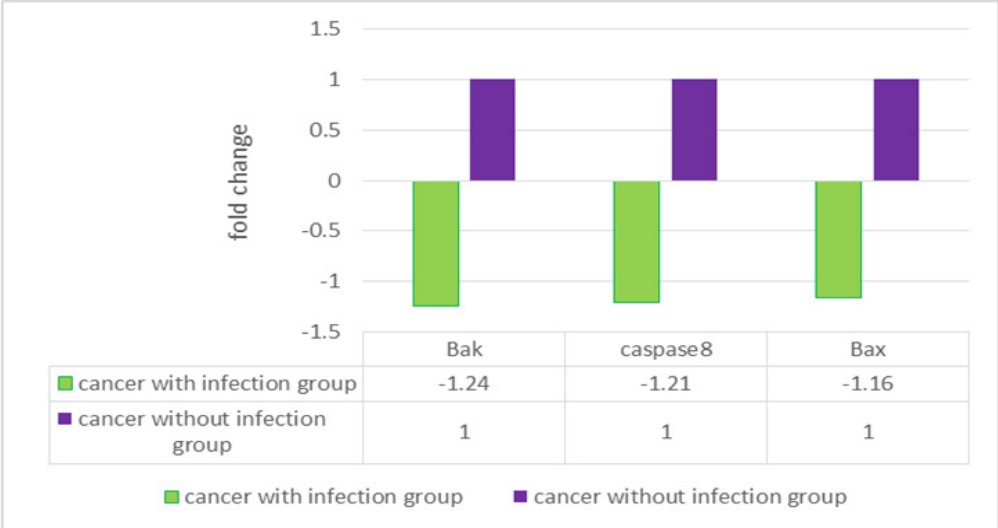


Figure 4. Expression Levels of *Bak, Bax,* and *Caspase 8* in Patients with Oral Infection and OSCC Patients without Oral Infection were Measured by RT-qPCR.

roles in vivo is required. Despite extensive research, there has been a lack of studies examining the role of miRNAs in OSCC. MiR-26 has been sparsely studied to date, and its relevance to the oncogenic process is only beginning to be elucidated. It has been shown that *miR-26a* is overexpressed in a subset of high-grade gliomas and directly targets the *PTEN* transcript [30]. Moreover, a previous study has reported a negative correlation between *PTEN* and *miR-26*a expression in patients with colorectal cancer. This suggests that *miR-26*a could potentially serve as a biomarker for tumor development [31]. Besides, growing evidence suggests that *miR-92* is another oncomir which highly overexpressed in several cancers in association with cancer development and progression. Some studies identified high expression of *miR-92a* in lung, breast, and colon cancers [32-34]. *MiR-92a* has been implicated in the suppression of the proapoptotic BH3-only protein Bim in human colorectal carcinoma [32]. Upon comparing the expression of *miR-92*a in benign breast tissue and breast cancer, it was found that *miR-92*a was upregulated in the most aggressive tumors exhibiting higher tumor grades and triple-negative receptor statuses [34]. In light of the role of *miR-26* and *miR-92* as an oncomir in cancer progression, our study has found that existence of pathogenic bacteria in the oral cavity can upregulate *miR-26* and *miR-92* expression in OSCC patients with oral infections.

In addition to the *miR-26* and *miR-92*, miR-486 is also involved in the development and progression of tumors and can function as both a tumor suppressor and an oncogene. The decreased expression of miR-486 has been observed to promote the progression of lung cancer, breast cancer, HCC, and osteosarcoma, while it is typically upregulated in pancreatic cancer, chronic myeloid leukemia, and gliomas [35]. It also suggested that downregulation of miR-486-5p contributes to tumor progression and metastasis by targeting protumor genic ARHGAP5 in lung cancer [36]. In this context, our study also exhibited that miR-486 expression was reduced in OSCC patients with oral infection. It appears that oral infection can lead to the progression of OSCC cancer through the downregulation of *miR-486* expression.

The Bcl2 family of genes plays a crucial role in the mitochondria-dependent pathway of apoptosis and is closely associated with the process of carcinogenesis [37]. Among the family members, *Bak* and *Bax* are death-promoting genes [38]. *Bcl-2* protein plays a key role in the process of gastric cancer formation and is associated with the growth of definite types of gastric cancer. Also, there is evidence that the *Bcl-2* protein plays a crucial role in the process of stomach cancer development and is associated with the growth of certain types of stomach cancer [39]. Furthermore, evidence suggested that overexpression of *Bax* induces apoptosis in lung cancer cell lines, but not normal cell lines [38]. Importantly, it has been shown that Chlamydia trachomatis infection inhibits both *Bax* and *Bak* activation. This observation has provided new information on the mechanisms of bacterial antiapoptotic activity [40]. Our findings are consistent with this evidence, showing that the presence of infection can lead to disruption of apoptosis and contribute to carcinogenesis by reducing the expression of *Bax* and *Bak*.

*Caspase-8* is an important caspase that triggers programmed cell death following the attachment of death receptors. Its critical role in apoptosis has resulted in significant medical interest in regulating *caspase-8* expression. However, Caspase-8 also performs various non-apoptotic functions in cells, including activating NF-kappaB signaling, controlling autophagy and changing endosomal trafficking, and improving cellular adhesion and movement [41]. As a result, depending on the particular cellular scenario, *caspase-8* may either boost or inhibit tumor malignancy [41]. Earlier research has revealed a correlation between the level of CASP8 expression and the tumor stage or disease prognosis. For instance, medulloblastoma patients with low CASP8 expression tend to have a poor prognosis. Similarly, a significant correlation between reduced CASP8 expression and the stage of HNSCC has been reported in previous studies [42, 43]. Furthermore, reduced expression of CASP8 has been demonstrated in breast cancer tissues when compared to adjacent normal tissues [44]. Given that caspase-3 activation is identified as the key biomarker of the apoptotic pathway, methylation status of *caspase-8* can affect the survival of human glioma cells by promoting anti-apoptotic mechanisms, which prevents the cells from undergoing programmed cell death. This can lead to the proliferation of cancerous cells and the progression of glioma [45]. CASP8 -652 6 N ins/del polymorphism affects patient susceptibility to multiple cancer types, including cancer of the oral squamous carcinoma, lungs, esophagus, stomach, colorectum, breast, and cervix in Chinese populations and cutaneous melanoma in Caucasian populations and may be used as a biomarker for this disease [41, 46]. It is intriguing to note that bacterial pathogens like Shigella can prevent *caspase-8* apoptosis when they invade the colonic epithelium during infection [47]. Parallel to previous evidence, the present study revealed that

oral pathogens can be involved in the prevention of apoptosis by reducing *caspase-8* in cancer cells and this mechanism can contribute to the development of cancerous tissue in patients with OSCC.

In Conclusion, these findings suggest that oral pathogens may be involved in the inhibition of apoptosis in cancer cells and play a significant role in tumorigenesis in OSCC tissue in affected patients. Understanding the mechanisms involved can help in developing new strategies for cancer prevention and treatment. Therefore, further studies are needed to investigate the role of oral microbiome in the development and progression of tumors in OSCC patients.

## Author Contribution Statement

Naghmeh Bahrami, Mohammad Bayat, Melika Norouzi, Zahra Gharayagh Zandi , Fereshteh Shirzadian contributed to the study design and manuscript drafting. Mehdi Kazempour dizaji, Abdolreza Mohamadnia , Mohammad Hossein Soltani, Farnaz Ahmadi , Shadi Shafaghi were responsible for data collection and analysis. Abdolreza Mohamadnia, Seyedeh Zahra Fotook Kiaei,

Mohammad Varahram contributed to the interpretation of findings. All authors reviewed and approved the final manuscript.

## Acknowledgements

*Availability of data*

The data supporting this study's findings are available from the corresponding author upon reasonable request.

*Ethics statement*

The study protocol was approved (ethical code: IR.SBMU.NRITLD.REC.1402.116)

*Conflict of interest*

The authors declare no conflicts of interest relevant to this study.

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