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Atorvastatin increases oxidative stress and inhibits cell migration of oral squamous cell carcinoma in vitro

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ABSTRACT

Objective: This study aimed to evaluate the effect of atorvastatin treatment on reactive oxygen species (ROS) production and tumor angiogenesis in oral squamous cell carcinomas.
Material and Methods: An HN13 cell line was treated with 1 μM, 5 μM, and 10 μM of atorvastatin. VEGF-A gene expression was evaluated by quantitative real time PCR. VEGF-A protein expression was quantified from total protein and conditioned media by ELISA. Cellular oxidative stress was measured using 2′,7′-dichlorfluorescein-diacetate (DCFH-DA). Angiogenesis assay was performed using human umbilical vein endothelial cells (HUVEC). The effect of atorvastatin on cell migration was evaluated by wound healing assay.
Results: 5 μM and 10 μM of atorvastatin significantly increased VEGF-A gene expression in the HN13 cell line. Intracellular expression of the VEGF-A protein was higher in the cells treated with 5 μM and 10 μM than in the control cells. VEGF-A protein expression was also higher in the conditioned media from the atorvastatin-treated cells than in the media from the DMSO-treated cells. 5 μM and 10 μM of atorvastatin increased oxidative stress. Regarding angiogenesis assay, 5 μM of atorvastatin resulted in higher numbers of branch points, compared to the solvent. 10 μM of atorvastatin treatment resulted in significantly reduced cell migration.
Conclusions: This study showed that atorvastatin increases the oxidative stress and angiogenesis in oral squamous cell carcinomas. The decrease of cell migration indicates atorvastatin's inhibitory effect in oral tumors. These results suggest that atorvastatin could increase the intracellular oxidative stress in these cells, leading to a toxic microenvironment and inhibiting their metastasis.

Introduction

Tumor cells exhibit an increase the levels of reactive oxygen species (ROS), which are involved in genetic instability, cancer progression, and redox regulation [1,2]. ROS bind to molecules responsible for signaling several cellular processes such as cellular proliferation, activated mitogenic protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways, ROS homeostasis, and regulation of antioxidant gene expression [3,4]. Oxidative stress occurs when ROS are not adequately neutralized or removed, and it is associated with a change in the intracellular ROS/antioxidant balance [5]. This imbalance results in an excess of ROS and can promote the formation of mutagenic compounds, activate proto-oncogenes, and deactivate tumor suppressor genes [6].

The intracellular accumulation of ROS induces cell cycle arresting at the G1 or G2/M phase, avoiding cell proliferation [7,8]. It is known that a moderate increase of ROS can contribute to cell differentiation and proliferation, but excessive ROS accumulation causes high oxidative stress and cell damage [8]. Malignant cells present an abnormal increase of ROS levels and need an efficient antioxidant system to maintain the redox balance for cell survival [9].

Evidence suggests that ROS production derived from mitochondrial metabolism or from the activity of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex induces VEGF-A gene expression and angiogenesis [10] that are associated with oral cancer [11]. The NADPH oxidase enzyme complex consists of two membrane-bound elements (gp91phox and p22phox), three cytosolic components (p67phox, p47phox, and p40phox), and a low-molecular-weight G protein (Rac1 or Rac2), which catalyzes the production of

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superoxide from oxygen and NADPH [12]. Rac1 protein, a member of the Rho GTPase family, is an intracellular transducer that regulates multiple signaling pathways responsible for controlling the cytoskeletal organization, cell transcription, and proliferation. Changes in Rac1 expression or activation may result in alteration of cell signaling and various pathological conditions [13].

Rac proteins from the NADPH oxidase enzyme complex can be inhibited by statins through the prevention of Rac translocation to the cell membrane [14]. Statins are drugs used to inhibit cholesterol biosynthesis by blocking the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase and preventing the conversion of HMG-CoA to mevalonate. Atorvastatin can inhibit the Rac1 activity, resulting in inhibition of the NADPH oxidase activity and ROS formation [15]. Atorvastatin was related to inhibition of NADPH oxidase activity and a decrease of VEGF-A expression in lung carcinoma cells [10]. Therefore, the role of ROS in the induction of angiogenesis through the increase of VEGF-A expression needs to be elucidated, and further studies are needed to understand the role of ROS metabolism in the carcinogenesis process. We know of no studies on ROS metabolism's role in the induction of VEGF-A expression and angiogenesis in head and neck cancer. Therefore, this study aimed to evaluate the effect of atorvastatin treatment on ROS production, tumor angiogenesis, and inhibition of oral squamous cell carcinomas.

Materials and Methods

Cell culture and reagents

The human oral squamous cell carcinoma cell line, HN13, was grown in a DMEM high glucose tissue culture medium with 10% fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were placed into tissue culture flasks and dishes and grown at 37 °C in a humidified atmosphere of 5% CO_2. Atorvastatin was provided by the Hospital de Base of Sao Jose do Rio Preto. Atorvastatin (Sigma Aldrich, Missouri, EUA) was dissolved in DMSO, and the final concentration of DMSO in all the experiments was 0.1%. After reaching 80% confluency, 2×10^5 cells were cultured in 6-well plates with culture media containing 1 μM, 5 μM, and 10 μM of atorvastatin or 0.1% DMSO (control) for 24 h in the subsequent experiments.

Determination of cell viability

The number of viable cells after Atorvastatin treatment was determined using trypan blue. Five microliters (μl) of the cell suspension were mixed with 45 μl of 0.4% trypan blue solution. The live and dead cells were counted under a light microscope, and the percentage of viable cells was calculated.

Evaluation of oxidative stress

Intracellular generation of ROS was measured using 2 ', 7′-dichlorfluorescein-diacetate (DCFH-DA), a fluorescent dye used for intracellular detection of hydrogen peroxide. After 24 h, the atorvastatin treated and non-treated cells were incubated with 5 μM of DCFH-DA for 30 min. The cells were washed, trypsinized, and resuspended in PBS. The mean fluorescence intensity at 530 nm was measured in the BD Flow Cytometry FACSCalibur (BD Biosciences, Nova Jersey, EUA) equipment.

Angiogenesis assay

Human umbilical vein endothelial cells (HUVEC) maintained in a complete medium were trypsinized and placed in 96-well plate containing the ECMatrixgel (Millipore, Massachusetts, EUA) and a conditioned culture medium from the HN13 cells treated with atorvastatin or DMSO (control). The cells were incubated in 5% CO_2 at 37 °C for 18 h. Six random fields per well were examined at 40X magnification, and the branch points were counted.

RNA and protein extraction from cell cultures

The extraction of RNA and proteins from the cultured atorvastatin- or DMSO-treated cells was performed using the Trizol reagent (Ambion, Austin, TX) and the RIPA buffer (Sigma Aldrich, Missouri, EUA), respectively. The samples were submitted to spectrophotometry (NanoDrop 200, Thermo Scientific, Massachusetts, EUA) for RNA quantification and quality evaluation. Complementary DNA (cDNA) was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Quantitative real time PCR

VEGF-A gene expression was evaluated using the Hs00900055_m1 Taqman gene expression assay (Applied Biosystems, Foster City, CA). Relative expression (fold change) of VEGF-A gene from the treated cells in relation to DMSO treated cells was calculated using the ΔΔCt algorithm [16]. The amplification reaction was performed according to manufacturer's instructions on the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA).

VEGF protein quantification by ELISA

Quantification of VEGF-A protein from the atorvastatin-treated and non-treated cells was performed by ELISA using Human VEGF DuoSet ELISA (R&D Systems, Minnesota, EUA) according to manufacturer's instructions. The VEGF-A protein concentration was determined in cell lysate and a conditioned medium obtained after the treatment of HN13 cells with various concentrations of atorvastatin or 0.1% DMSO.

Migration assay

The effect of atorvastatin on cell migration was evaluated by wound healing assay. The cells were seeded in 6-well plates containing a complete medium and incubated at 37 °C in an atmosphere of 5% CO_2. At about 90% confluence, a fine scratch was made with a pipette tip, and the complete medium was replaced with a serum-free medium in the presence of 1 μM, 5 μM, and 10 μM of atorvastatin or 0.1% DMSO (control) for 24 h. The scratch's width was measured at 0 and 24 h to calculate the percentage of the area covered by the cells.

Statistical analyses

Gene quantification analysis was performed by a one-sample *t* Test. A two-sample *t* Test and analysis of variance (ANOVA) were used for analysis of differences between the groups, such as protein expression, ROS quantification, angiogenesis, and cell migration. Statistical analyses were performed using the GraphPad Prism software version 5.01. Results with a p value < 0.05 were considered significant.

Results

Effect of atorvastatin on cell viability of oral squamous cell carcinomas

No significant alteration of cell viability was observed with 1 μM (83.9%), 5 μM (75.2%), or 10 μM (73.4%) of atorvastatin compared to 0.1% DMSO (96.9%). The numbers of viable cells were analyzed by ANOVA with the Tukey-Kramer multiple comparison test ($p = 0.22$).

Atorvastatin increases oxidative stress in oral squamous cell carcinomas

To analyze atorvastatin's effect on ROS production, we used DCFH-

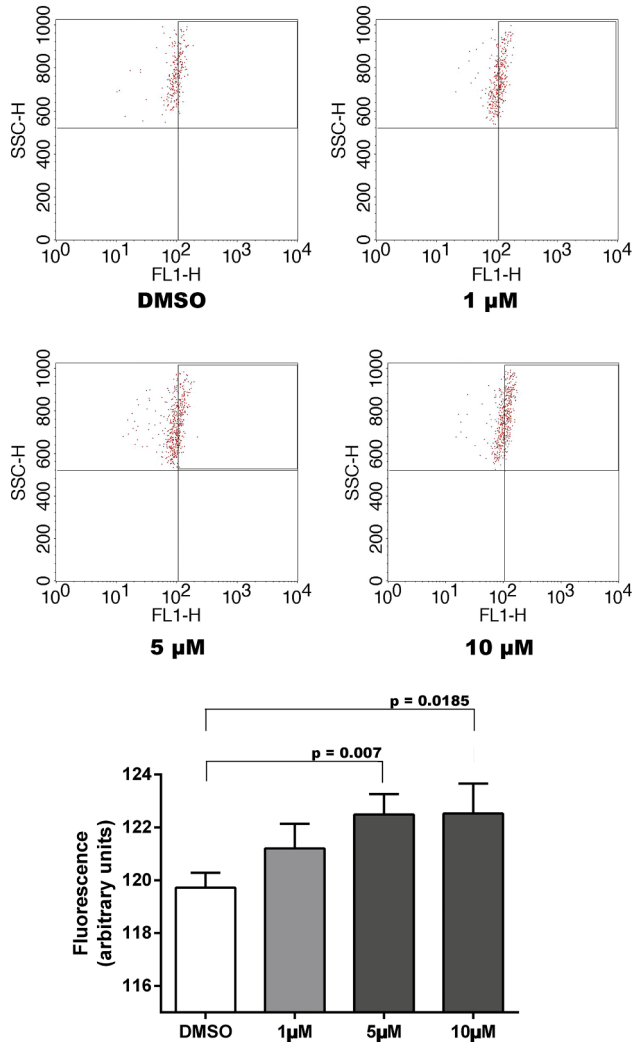

Fig. 1. 2′,7-dichlorofluorescein-DCF fluorescence from atorvastatin-treated HN13 cells. 5 μM and 10 μM of atorvastatin significantly increased ROS production in oral squamous cell carcinomas, compared to the DMSO control. Each value represents mean ± S.E. from three separate experiments. Statistical analysis by unpaired *t* test.

DA, a fluorescent dye able to detect intracellular hydrogen peroxide. The dye penetrates the cells and is hydrolyzed to DCFH by cell esterase. DCFH is oxidized by ROS to a highly fluorescent compound (2′,7-dichlorofluorescein-DCF). As shown in Fig. 1, the treatment of the NH13 cells with atorvastatin for 24 h resulted in increased ROS production demonstrated by the high intensity of DCF fluorescence. Atorvastatin increased oxidative stress in NH13 cells at 5 μM ($p = 0.007$) and 10 μM ($p = 0.018$).

Atorvastatin increases angiogenesis in oral squamous cell carcinomas

HUVEC cultured with a conditioned medium from the HN13 cells treated with 5 μM of atorvastatin resulted in more vessels than those incubated with DMSO ($p = 0.0047$) (Fig. 2).

VEGF-A gene and protein expression is increased in atorvastatin-treated oral squamous cell carcinomas

The 5-μM and 10-μM atorvastatin treatments significantly increased VEGF-A gene expression in the HN13 cell line (RQ = 1.4, $p < 0.0001$ and RQ = 1.9, $p < 0.0001$, respectively) (Fig. 3). Intracellular expression (from total protein extracted from the cells) of the VEGF-A protein was higher in the 5-μM (16.4 μg/mL, $p = 0.018$) and 10-μM (15.6 μg/mL, $p = 0.029$) atorvastatin-treated cells than in the control cells (10.3 μg/mL) (Fig. 4). The expression of VEGF-A proteins was also higher in the conditioned media from the atorvastatin-treated cells (5 μM: 38.7 μg/mL, $p = 0.013$; 10 μM: 37.9 μg/mL, $p = 0.0018$) than in the control cells (10.7 μg/mL) (Fig. 4).

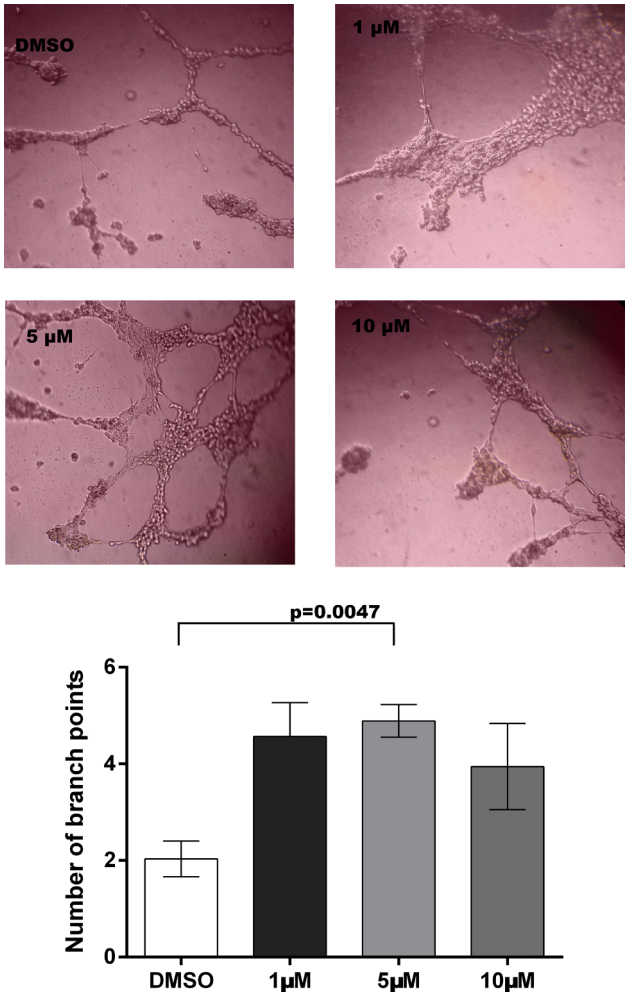

Fig. 2. Effect of atorvastatin on HUVEC cell tube formation. HUVEC cells were atorvastatin- or DMSO-treated for 18 h. The ability of tube formation in 5 μM-atorvastatin-treated cells was significantly higher than that in DMSO-treated cells. Each value represents mean ± S.E. from three separate experiments. Statistical analysis by unpaired *t* test. 40X magnification.

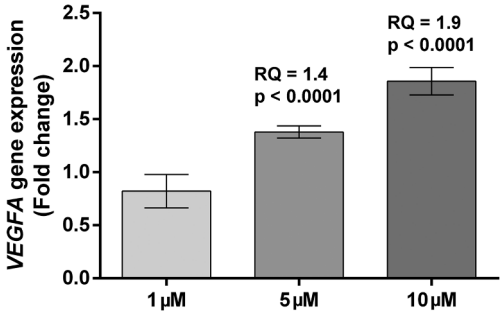

Fig. 3. VEGF-A gene expression in atorvastatin-treated oral squamous cell carcinomas, compared to DMSO-treated cells. Fold changes were Log2 transformed (y-axis). VEGF-A was overexpressed in cells treated with 5 μM-and 10 μM of atorvastatin for 24 h (one-sample test: $p < 0.0001$). The bars represent mean ± S.E. Calibrator (DMSO-treated cell) log $RQ = 0$.

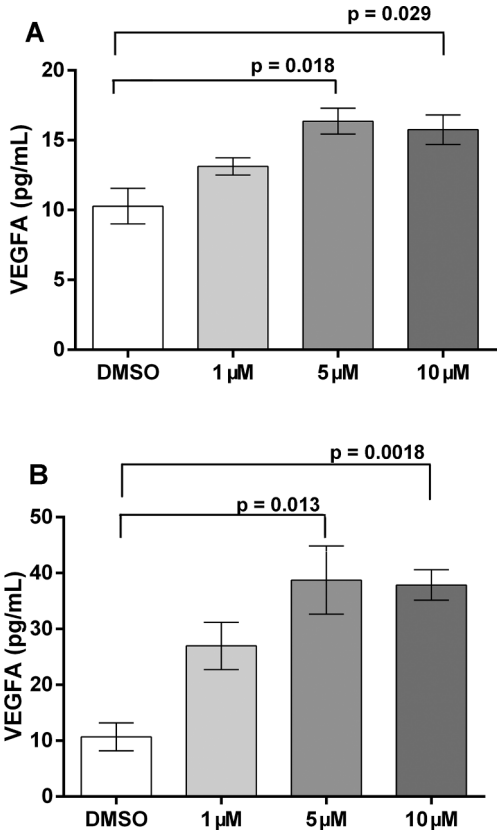

Fig. 4. A. Effect of atorvastatin on HN13 intracellular VEGF-A protein levels. Intracellular VEGF-A protein levels from HN13 cells treated with 5 µM (16.4 µg/mL) and 10 µM (15.6 µg/mL) of atorvastatin were significantly higher than those from DMSO-treated cells (10.3 µg/mL). **B. Effect of atorvastatin on VEGF-A protein levels from HN13 conditioned medium.** VEGF-A protein levels obtained from a conditioned medium of HN13 treated with 5 µM (38.7 µg/mL) and 10 µM (37.9 µg/mL) of atorvastatin were significantly higher than those from DMSO-treated cells (10.7 µg/mL). Each value represents mean ± S.E. from three separate experiments. Statistical analysis by unpaired *t* test.

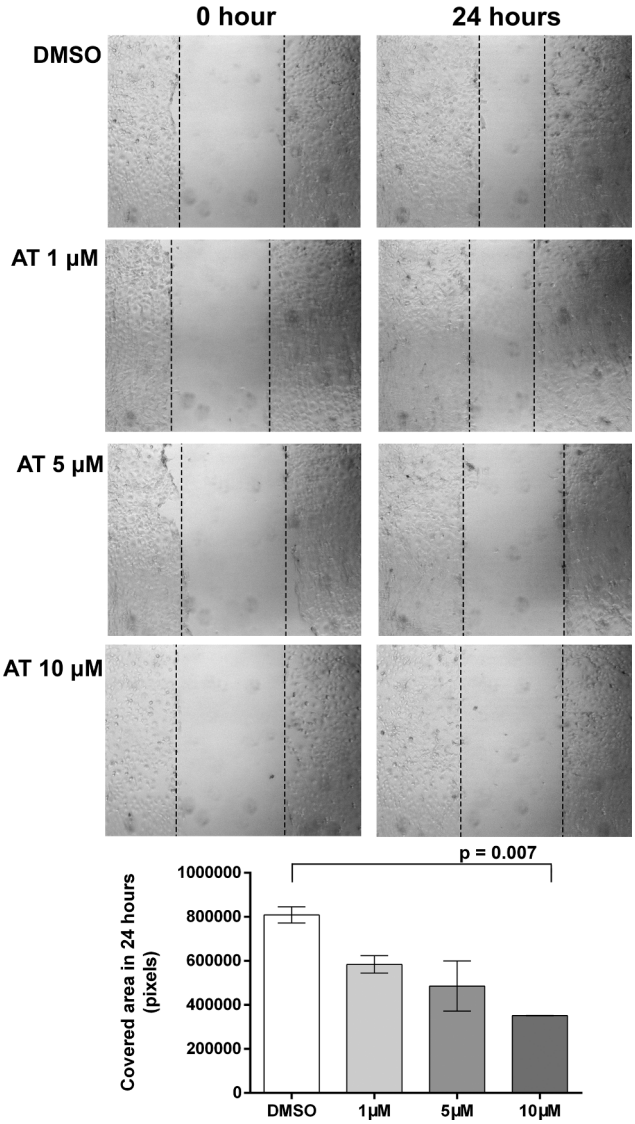

Fig. 5. Effect of atorvastatin on HN13 cell migration. After 24 h, the migration of the 10-µM atorvastatin-treated cells was significantly reduced, compared to the DMSO-treated cells. Each value represents mean ± S.E. from three separate experiments. Statistical analysis by unpaired *t* test. 100X magnification. AT: atorvastatin.

Atorvastatin reduces cell migration

At about 90% confluence, a scratch wound was created, and subsequent observation was conducted. At the 24th hour, the wound closure ability had significantly decreased in the atorvastatin-treated cells compared to the DMSO-treated cells (Fig. 5). The 10-µM atorvastatin treatment resulted in significantly reduced cell migration ($p = 0.0064$) and evidenced an inhibitory effect on the oral tumor cells.

Discussion

The present study showed an inhibitory effect of atorvastatin on oral squamous cell carcinomas, evidenced through the decrease of cell migration after treatment with high-dose atorvastatin. Researchers have shown that statins act as inhibitors of cell proliferation by inducing apoptosis in several cancer types and that they prevent cell invasion and metastasis in various cancers [17,18].

The atorvastatin treatment's inhibition of migration and invasion was observed in the squamous cells carcinoma SCC-1 and SCC-47 cell lines, derived from the floor of the mouth and the tongue, respectively [19]. The study suggested that atorvastatin may control metastasis through the decrease of RhoC's functional activity (a GTPase belonging to the Ras superfamily), which results in the diminished directional movement of head and neck cancer cells and consequently the prevention of metastasis. A significant decrease in active RhoC was observed in these atorvastatin-treated cell lines. In an in vivo animal model, RhoC inhibition also resulted in an inhibition of metastases, compared to the placebo-treated animals [19].

Atorvastatin was associated with the expression of cell cytoskeletons in endothelial cells [20]. However, few studies demonstrate the relationship between atorvastatin and lamellipodia. One study suggested that atorvastatin decreases F/G actin and the expression of F-actin in lamellipodia, which may correlate with the attenuation of cell migration and angiogenesis. According to this previous study, atorvastatin decreased migration and tube formation in endothelial cells involving receptor-related urokinase factors, AP5, p-FAK, Rac, and reorganization of F-actin cytoskeletons [21].

The biphasic effects of statins on angiogenesis have been observed and have been ascribed to opposite actions of low versus high drug concentrations [22,23]. At the molecular level, statins' protective activities are mostly ascribed to stimulation of the PI3-Akt kinase pathway, resulting in phosphorylation of eNOS, a critical mediator of angiogenic and anti-apoptotic activity in endothelial cells [24,25]. On

the other hand, higher concentrations of statins may exert weak or no effects on Akt kinase phosphorylation [25].

Some studies have shown that statins activate angiogenesis by promoting migration and proliferation of endothelial progenitor cells derived from bone marrow and tissue in the foci of ischemia and neovascularization [26]. Although not entirely understood, Akt signaling is central in statin-mediated angiogenesis by promoting endothelial cell survival, inhibiting apoptosis, activating eNOS, and increasing nitric oxide bioavailability [27].

In cancer, atorvastatin inhibited VEGF-A expression in non-small cell lung cancer in vitro and in vivo via inhibition of ROS production. In these cells, atorvastatin inhibited ROS formation through the suppression of Rac1/NADPH oxidase activity [10]. On the other hand, in the present study, atorvastatin treatment at 5 μM and 10 μM significantly increased VEGF-A gene and protein expression in the HN13 cell line. Furthermore, high-dose atorvastatin (5 μM) resulted in increased angiogenesis, different from the literature on other cancer types [28]. Similar to our study, treatment of breast cancer cell lines with increasing doses of atorvastatin resulted in high levels of VEGF production via inhibited geranylgeranylation [29].

VEGF-A not only induces vascularization but also acts as a direct survival factor for tumor cells [30]. VEGF-A has been shown to prevent radiation-induced apoptosis and to up- regulate Bcl-2 [31,32]. However, anti-angiogenic treatment has been associated with increased metastases and relapses after use [33,34]. Anti-angiogenic drugs can also modify the tumor's microenvironment by creating hypoxic niches within the tumor mass. Indeed, the development of hypoxic niches in breast cancer treated with anti-angiogenic molecules resulted in the accumulation of cancer stem cells (CSCs) [35].

CSCs are involved in aggressive tumor behavior, resistance to chemotherapy, and an enhanced ability to adapt to changes in the tumor's microenvironment. So the stimulation of CSCs after anti-angiogenic treatment may provide a rationale for a combined treatment with other effective drugs against tumor development. In colorectal cancer, 0.1 μM of atorvastatin in combination with bevacizumab reduced HUVEC's cell viability, migration, invasion, and tube formation [36]. The combination of docetaxel with atorvastatin potently inhibited growth and induced apoptosis in prostate cell lines. A mechanistic study indicated that the induction of apoptosis in PC-3 cells was associated with a significant decrease in the levels of Bcl-2, VEGF-A, phosphor-Akt, and phosphor-Erk1/2 [37].

ROS production is closely related to VEGF-A expression and secretion in various cardiovascular diseases [38,39]. The NADPH oxidase/ROS pathway has also been demonstrated to induce the VEGF-A expression in human ovarian cancer cells and human non-small cell lung carcinoma [10,40]. The NADPH oxidase enzyme complex consists of two membrane-bound elements (gp91phox and p22phox), three cytosolic components (p67phox, p47phox, and p40phox) and a low–-molecular-weight G protein (Rac 1 or Rac2) and catalyzes the production of superoxide from oxygen and NADPH [12]. Inhibition of ROS production by antioxidant agents, such as NAC (N-acetylcystein) and DPI (Diphenylene iodonium), or diminishing of the expression of the p47phox protein, a cytosolic component of the NADPH oxidase enzyme complex, can effectively block VEGF-A expression in ovarian tumor cells [40].

Our results showed that atorvastatin increased ROS production in oral tumors, which is probably related to the VEGF-A overexpression and increased angiogenesis. However, atorvastatin have been reported to have antioxidant properties [10]. Thus, the effect of atorvastatin on oxidative stress has yet to be elucidated. One cause for increased oxidative stress could be the reduction of coenzyme Q10 by atorvastatin, resulting in redox imbalance toward oxidation [41]. Coenzyme Q10 has a protective role by inhibiting lipid peroxidation [42,43]. The increased angiogenesis is associated with the development of metastasis in several tumor types [44]. In the present study, this increase appears to be a result of increased oxidative stress and may not be a negative factor in oral cancer once cell migration was reduced. The next point to be elucidated is the effect of atorvastatin in CSCs, considering the behavior of this cell type to the reduction of angiogenesis previously committed.

Despite the action of ROS in cancer initiation, it is supposed that increased oxidative stress by ROS generation therapy kills cancer cells without damaging normal cells. Researchers have found that 10–100 μM of hydrogen peroxide can kill apoptotic cells [45]. A substantial increase of ROS causes irreversible damage to cells, resulting in cell death [8]. Therefore, these biological features of ROS make possible their use to eliminate tumor cells.

Cancer cells may be more sensitive than normal cells to an increase of ROS, which offers an interesting therapeutic way [46]. Hence, increasing ROS to reach a threshold that is incompatible with cell viability and targeting the enhanced antioxidant mechanisms can selectively kill tumor cells without affecting normal cells [47,48]. Despite the reduced ROS levels in CSCs and the efficient detoxifying systems, increased ROS concentrations can still eliminate these cells [45]. Several chemotherapeutic agents are currently used to induce ROS production, resulting in cell death [49–51]. In breast cancer cells, paclitaxel promotes ROS formation through the translocation of Rac1, which positively regulates the activity of NADPH oxidase. Currently, radiotherapy is widely used in several cancer treatments, mainly depending on ROS generation [52–55]. Therefore, the use of atorvastatin in the treatment of oral cancer could be an option to avoid the high recurrence rate after the treatments currently used.In summary, the present study showed that atorvastatin increases the oxidative stress and angiogenesis in oral squamous cell carcinomas. The decrease of cell migration indicates atorvastatin's inhibitory effect in oral tumors. These results suggest that atorvastatin could increase these cells' intracellular oxidative stress, leading to a toxic microenvironment and inhibiting their metastasis.

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Conflict of interest statement

None declared.

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