Levofloxacin might be safe to use for OSCC patients

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Abstract
Oral squamous cell carcinoma patients are exhausted against the powerful chemotherapies, radiotherapies after the surgery, and their immune system is devastated during the process and antibiotic usage become inescapable. Although prescribing an antibiotic might be fraught for such as drug interaction and undesirable proliferation danger, studies still look for the new ideas such as antibiotic combinations that might be safe to use. The antiproliferative and apoptotic outcomes of levofloxacin with cisplatin combination as well as their single usage were examined with WST-1, Caspase-3/BCA and Annexin V methods on SCC-15 cells and a healthy cell line (MRC-5). 24 h treatment of 50 mM single levofloxacin, 50 mM single cisplatin and 50 mM levofloxacin-cisplatin combination resulted in viability rates of SCC-15 cells as 90%, 67% and 80.8%, respectively. Caspase-3 enzyme activity was enhanced 0.92-fold for single levofloxacin, 13.05-fold for single cisplatin and 9.73-fold for the combination of levofloxacin-cisplatin, the total apoptotic activity of single levofloxacin, single cisplatin and levofloxacin-cisplatin combination were observed as 4.88%, 21.14%, 16.21%, respectively on SCC-15. The apoptotic effect of cisplatin on MRC-5 has been shown to be suppressed when combined with levofloxacin. Considering the cell viability, caspase-3, and apoptotic activity results, it’s conclude that the levofloxacin-cisplatin combination was also effective compared to the only cisplatin treatment on OSCC cells. The combination has shown less toxicity for healthy cells than single cisplatin treatment. Therefore, our apoptotic findings suggest that the different dosage combinations are necessary to understand the interaction for the treatment of tongue squamous cell carcinoma.

Keywords Oral squamous cell carcinoma · Levofloxacin · Cisplatin

Introduction
Oral cavity cancers have been following the top-ten common cancer in the world, and they include epidermoid carcinomas, salivary gland carcinomas, lymphomas, sarcomas, and melanomas [1]. Approximately 90% of oral cavity cancers are diagnosed as oral squamous cell type. Oral squamous cell carcinoma (OSCC), the most common malign type of oral cavities, is the sixth most common cancer in the world and highly metastatic for head and neck cancers as a subgroup of it [2]. Some life choices such as smoking and alcohol are the primary risk factors for OSCC moreover, human papillomavirus, genetic tendency and life conditions also contributing factors for the disease as well as in head and neck carcinomas. Excluding the genetic factors, having risk for OSCC is highly on individual's hand however, the world is rolling around with a lot of people who are not choosing to change their habits for their health, respectfully. That is the reason, we presume that OSCC would probably be a big health issue due to the capacity of being on top for cancer cases. The predict idea about OSCC danger in future; the new perspectives, new ideas, new available options, even alternatives for decreasing the side effects on the treatment options are important for the literature.
OSCC derives from epithelial squamous, including parts such as the tongue, buccal surface, floor of the mouth, soft and hard palates [3, 4]. The tongue type OSCC risk for young adult patients is increasing rapidly and is expected to increase more in the future [5]. Tumor localization in the tongue is a common occurrence and resulting in very aggressive outcomes for patients with OSCC. The tongue and predominantly lateral part of it with the left, right, anterior and posterior parts set forth an important majority of intraoral cancers [6]. The tongue-rooted carcinomas establish serious substructure for head and neck metastasis. Metastasis from tongue to the head and neck, and the presence of fixed ganglions are the signs for the poor prognosis [7]. Not only because tumor localization in the tongue has the aggressive capacity, but also it has the perfect ability to metastasize on neighbor tissues and organs very quickly.

Tumor classification is generally based on localization of it, histological grade and TNM (T: Primary tumor size, N: Neck involvement, M: metastasis) staging. Unfortunately, diagnosing the patient in an early stage with OSCC is really rough since no symptoms until the disease get advanced. As a consequence of this, the 5-year survival rate for OSCC is desperately low in patients regardless of the treatment [7, 8]. In the early stages of the disease, surgical excisions might provide a good survival rate. On the other hand, in addition to decreased level of survival in advanced-stage tumors, receiving radiotherapy and chemotherapy with more and prolonged side effects might be exhaustive for patients [9]. Cisplatin is one of the best effective chemotherapeutic agents and widely used in many cancer treatments including the OSCC [10] with still controversial utilization in OSCC treatment because of acquired drug resistance and side effects in general cancer treatments [11].

Nevertheless, fighting with cancer make the body vulnerable to infections either with side effects or immunity changes. Using the proper antibiotic with the right dose in the line of rational drug use (RDU) is significantly important for the people on this stage of their life. When antibiotic becomes necessary for patients, the expectations are just proper help for the infections and decreasing the side effects of chemotherapeutics. Other than that, it might be too much work for the body’s system by loading drugs to metabolize without any benefits. Although, antibiotics are coming from the ancient times and still on the table for their fight and success with infections including some concerns about antibiotic’s safe usage for cancer patients, they have also been rising out with a curious manner on cancer treatment too. Recently combined drug treatment with low doses of chemotherapeutics in cancer patients has become very popular [12] to diminish or even repress the side effects at the same time treatment success on it’s way including the remain healthy cells still are not/even less toxically effected during the process. Antibiotics have been used in different cancer treatment researches for investigations of their antiproliferative and apoptotic effects [13, 14]. This is because some antibiotics have been shown in recent studies to be effective not only in inhibiting bacterial growth but also in stimulating immunomodulation [14, 15].

Fluoroquinolones, which have been used as antibiotics for more than four decades, suppress replication of bacterial DNA either by targeting DNA gyrase and/or topoisomerase II [16]. In addition to the immunomodulatory activities of fluoroquinolones, such as supporting anti-inflammatory response and cytokine production, their anticancer potential has also been under investigation in the last few years. Recent studies have shown that fluoroquinolones perform a supportive function for anti-proliferative, pro-apoptotic, and anti-metastatic activities due to their different pharmacological properties [14]. Levofloxacin is a fluoroquinolone antibiotic that has the area to treat upper airway-related diseases and for many bacterial infections in the body [17]. Levofloxacin is a pyridone carboxylic acid derivative and is used as a wide-spectrum antibiotic due to its distribution throughout the body and its strong intracellular penetration capabilities for both gram-positive and gram-negative bacteria [18]. There are also studies investigating the potential anticancer activity of levofloxacin when administered with different techniques [19–24]. Yu et al. showed that levofloxacin selectively inhibits cancer cell proliferation in breast cancer cell lines and works synergistically with 5-Fluorouracil (5-FU), a conventionally used chemotherapeutic agent. It was also reported that levofloxacin works through inhibition of mitochondrial biogenesis and stimulates the apoptotic panel in breast cancer cells while protecting healthy breast cells [24]. In another study, anti-proliferative and pro-apoptotic activities of levofloxacin were demonstrated using lung cancer cell lines and xenograft lung tumor model [22]. On the other hand, a number of studies have suggested that levofloxacin derivatives also exhibit anticancer activity on different types of cancer [25, 26]. The hydrazine derivative of levofloxacin has been shown to selectively suppress proliferation of cancer cells in a time- and dose-dependent manner in hepatocellular carcinoma cells and induce apoptosis through caspase cascade activation [26].

Another dilemma to find an answer for the usage of antibiotics in cancer patients is drug interactions. A variety of infectious pathogens such; Helicobacter pylori, Fusobacterium spp, Human papillomavirus, Streptococcus Bovis in patients with different cancer types are often encountered. These infections are experienced more resistently by cancer patients and their treatment requires wide-spectrum antibiotics, mostly a combination of a couple [27, 28]. Since these patients are already being treated with a set of chemotherapeutics, clinicians have doubts when prescribing any additional drug due to the risk of potential drug interactions.
Besides, these immunocompromised cancer patients easily suffer from the side effects of strong chemotherapeutics [29]. Prescribing antibiotics with several doubts for cancer patients is needed to be enlightened eventually in the line of rapid diagnostic unit with preliminary researches to find an answer for further analysis. And all, searching a method for using less concentrated chemotherapeutics but still effective combinations for cancer cells might be important for decreasing the side effects at the same time. The questions are still on going for their contribution on the field, and results are accumulating with the researches. With these promising points and empties in the literature for tongue type OSCC which has rapidly growing rate in all cancer cases, we have aimed to find some answers for the combination therapy between levofloxacin as an upper airways targeting antibiotic as well as it’s single usage and cisplatin as clinically common chemotherapeutic agent on SCC-15 cell line as tongue typed OSCC.

Materials and methods

SCC-15 (CRL-1623) tongue squamous cell line (LOT: 63,087,053) and MRC-5 (CCL-171) healthy lung cell line (LOT: 63,405,646) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). For the SCC-15 cells, 1:1(v/v) mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium supplemented with 400 ng/ml hydrocortisone and 10% Fetal Bovine Serum (FBS) was used. MRC-5 cells were seeded in Eagle’s Minimal Essential Medium (EMEM) supplemented with 10% FBS. Incubation conditions were kept as 37 °C and 5% CO₂ for both cells. As we think that levofloxacin activity may be affected by any other antibiotic activity, we did not add any other standard anticontamination antibiotic (penicillin–streptomycin, gentamicin, et cetera) in our standard mediums for all experiments.

Chemicals and assays

Dimethyl sulfoxide (DMSO) (D4540, 500 ml), and Cisplatin (CAS 15663) were purchased from Sigma Aldrich (St. Louis, USA), then cisplatin dissolved in DMSO. Levofloxacin (Floxielevo-750 mg) powders were weighed and dissolved in DMSO as a stock solution. Dulbecco’s Phosphate-Buffered Saline (DPBS) (Catalog Number: 14190367-Ca⁺², and Mg⁺² free) was obtained from Gibco Life Technoligies (Grand Island, MO, USA). Trypsin-Ethylendiaminetetraacetic acid-(Trypsin–EDTA, Catalog Number: 25200072) from Gibco was used for subculture experiments. WST-1 (Catalog Number: 11644807001) kit from Roche Life Sciences (Germany) was used to determine the cell viability. The caspase-3 colorimetric kit (Catalog Number: K106-100) was purchased from BioVision Research Products (Milpitas, CA, USA). Apoptotic activity was determined with Muse Annexin V-FITC (Catalog Number: MCH100105 EMD Millipore) and purchased from Merck Millipore (Burlington, MA, USA).

Subculturing and WST-1 viability assays

SCC-15 and MRC-5 cell lines were seeded and grown in 75 cm² flasks. Following the every 3 days, fresh mediums were added to the flasks. As cells reach the confluent phase in the flasks, we detached them for experiments. For each well of 96-well plate, 0.4 × 10⁵ cells were added in and then plates were put in an incubator. Following 24 h incubation step, cells were treated with determined dosages of levofloxacin and cisplatin in between 5 and 100 mmol, each for 24, 48, and 72 h. Cisplatin and levofloxacin treated cells were worked with WST-1 assay kit to specify their cell viability. After each incubation period, 10 μl WST-1 solution were added to each 96-well plates of SCC-15 and MRC-5 to determine the levofloxacin and cisplatin effects on cell viability. Cell viability was determined following 4 h of WST-1 incubation period and each wells were read at 440 nm using Multiscan ELISA reader (Thermo Fisher Scientific, Germany). All experiments were made three times, and the mean of three replicates was used.

Caspase-3 colorimetric assay

SCC-15 and MRC-5 were seeded in 6-well plates for 1.5 × 10⁶ cells per well. Different doses of each drug were added to the specific wells and incubated for 24 h. In the next step, treated cells were gathered for experiments. For each step, cells were treated with determined dosages of levofloxacin and cisplatin in between 5 and 100 mmol, each for 24, 48, and 72 h. Cisplatin and levofloxacin treated cells were worked with WST-1 assay kit to specify their cell viability. Cell viability was determined following 4 h of WST-1 incubation period and each wells were read at 440 nm using Multiscan ELISA reader (Thermo Fisher Scientific, Germany). All experiments were made three times, and the mean of three replicates was used.

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Determining the apoptotic activity with Muse Annexin V-FITC kit

Apoptotic activities of the cells were determined by measuring the DNA content of each cells on a fluorescence activated FITC-conjugated Annexin V-lectin in Muse annexin analyser device. This method is based on detection of the phosphatidylserine on the cell surface of the apoptotic cells after treatments for apoptotic sign. First, 0.1 × 10⁶ cells were seeded in 6-well plates for each well, then every treatment group wells were treated with varying drug doses. The SCC-15 and MRC-5 cells were treated with three different groups on 24 h time period; 50 mM doses of levofloxacin (Levo), 50 mM cisplatin (Cis) and combination of these two drugs (Levo-Cis). Following the incubation period, cells were centrifuged and supernatants were removed. The binding buffers were added by homogenization way. 5 µl FITC-Annexin V and same amount of PI reagents were added on cells. Following the vortex step, cells were kept at 20–25 °C and kept from light for 15 min. Results were measured in a flow cytometry device. For each two cell group for all treatment; live cell, dead cell, early and the late apoptosis cells were identified.

Statistical analysis

All experiments were made three times, and the mean of three replicates was used. GraphPad PRISM v 7.04 program was used to data analyze and creating the graphs (GraphPad Software, Inc. CA, USA). The statistical test information was included in each graph sub-text. All P-values resulted from two-sided statistical tests and P < 0.05 was considered to indicate a statistically significant difference.

Results

Levofoxacin-cisplatin combination enhances the cisplatin activity on SCC-15, also reduces the cisplatin toxicity on MRC-5 cells

Cell viability was assessed for all concentrations due to the time and dose-depended manner including single usage of levofloxacin, single usage of cisplatin and also their combinations with WST-1. Single levofloxacin treatment on SCC-15 cells has not altered the cell viability in all concentrations and time points that we have examined. In contrast to this, single cisplatin treatment on SCC-15 cells has been ended up with the inhibition impact on cell proliferation, as expected due to the clinical usage. After each drug and their combinations were administered for the MRC-5 as a healthy cell, the results were toxic in these non-cancer cells on 48 h and 72 h incubation periods. When the time was increased the toxicity has become more effective for all cells. Considering this, for 24 h and 50 mmol dose treatment with two different drugs and their combination results were the most effective one not only for antiproliferative impact on SCC-15 but also less toxic impact for MRC-5.

After 24 h single cisplatin treatment, cell viability results on SCC-15 were found as 67% when compared to the control cells which were untreated with any cisplatin dosage (Fig. 1). In addition to this, single levofloxacin treatment in same period was considered as having the lowest toxicity with the 90% rate of on the SCC-15 cell viability. When their combination was tested in 24 h, results revealed that cell viability was increased to the level of 80.8% on SCC-15.

The toxic effect of levofloxacin and cisplatin combination was reduced the toxicity on cancer and healthy cells.

Fig. 1 The cell viability for each drug and combinations on SCC-15 cells that were represented on connecting line groups, each shape represents the one drug dose. Dunnett’s multiple comparison test was performed in GraphPad PRISM v 7.04. The error bars represent mean ± standard deviation.
in comparison with single cisplatin usage even though single levofloxacin had 10% of toxicity. Also, combination results have less cytotoxic effect on MRC-5 than SCC-15 cells (Fig. 2). Looking with toxicity perspective, after the drug interaction, toxicity rates were found 10% for single levofloxacin, 33% for single cisplatin and 19.2% for their combination. After these findings, we have examined the Caspase-3 / BCA and Annexin V for understanding the cell death reason.

**Caspase-3/BCA enzymatic activity results correlated with cell viability results**

The caspase-3 activity of levofloxacin and cisplatin treated SCC-15, and MRC-5 cells were analysed with caspase-3 BioVision kit and BCA protein assay kit to determine the total protein concentration of cells. Following this step, caspase-3/BCA protein results were calculated. After 24 h treatment, our results have showed no significant caspase-3/BCA activity with 0.92-fold for single levofloxacin treatment on SCC-15 cells. In addition to this, Caspase-3/BCA activity was found increased by 13.05-fold with 50 mM single cisplatin and 9.73-fold with 50 mM levofloxacin-cisplatin combination on SCC-15 cells (Fig. 3). And gives us again the supportive outcomes that the results were showing that single levofloxacin usage might be safe for patients. Combination results showed that the levofloxacin was contributed by decreasing the cisplatin effect when administered together than single cisplatin effect. The caspase-3/BCA activity was not found to be significant based on the results of single or drug combination treatment for 24 h on MRC-5 cells.

**Apoptotic activity**

For each group, untreated cells were specified and compared as control groups. Only levofloxacin treatment has showed the apoptotic activity rate of 4.35%, the combination rate of 15.88% while only cisplatin rate of 22.05% on MRC-5. Only levofloxacin treatment, has not significantly altered the apoptotic activity on SCC-15 cells with the rate of 4.88%. But levofloxacin and cisplatin combination was resulted by apoptotic activity with the total rate of 16.21% while single cisplatin was resulted with the total rate of 21.14% on SCC-15 (Fig. 4).
Cisplatin, which is widely used in the treatment of many cancer types due to its high clinical efficacy, is a chemotherapeutic agent that acts by forming intra-strand cross-links in the DNA strand [34]. In many studies, it has been determined that cisplatin showed anticancer activity in many solid tumors such as lung, ovarian and breast cancer [35–37]. Our study has showed the cisplatin-induced apoptosis and inhibited cell proliferation in tongue squamous cell carcinoma cell line too. Despite its strong antiproliferative effect, various studies have shown that cisplatin treatment with high doses caused various side effects in patients such as nephrotoxicity, ototoxicity and hepatotoxicity. As a clinical reflection of its side effects, cisplatin caused an increase in blood urea, nitrogen, and creatinine levels and pathological changes of liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) in time-dependent manner [38]. The most common side effects of cisplatin are nausea and vomiting due to its toxicity on the gastrointestinal system [34, 39].

Given the toxic effects of chemotherapeutic agents on healthy cells due to the use of high doses especially in more advanced tumors, it has become necessary to investigate different methods to minimize these effects of the agents. In this context, low-dose combination of chemotherapeutics, additional natural compounds or certain antibiotics for treatments are considered as new options that may be beneficial [40]. Particularly, antibiotics have been reported to have anticancer activities, similar to their effects on bacteria in treatment of bacterial infections [41]. Levofloxacin, one of the antibiotics with anticancer activity, is a second-generation fluoroquinolone used in the treatment of upper respiratory tract-related infections. The anticancer activity of levofloxacin has been frequently investigated recently, effects such as suppression of proliferation, regulation of apoptosis, and reduction of ATP production by mitochondrial electron chain damage have all been demonstrated in lung cancer cells [22]. In breast cancer cells, levofloxacin inhibited mitochondrial biogenesis via suppression of major survival pathways PI3K/Akt/mTOR and MAPK/ERK. In the xenograft mouse breast cancer models, the combination of 5-Fluorouracil (5-FU) and levofloxacin synergistically inhibited tumor growth compared to 5-FU monotherapy [24]. In another study, researchers evaluated the anticancer activities of 15 acidic drugs selected from eight classes, including fluoroquinolones and nonsteroidal anti-inflammatory agents, on different cancer cell lines and reported that levofloxacin has selective cytotoxic activity on cancer cells. According to the study, while levofloxacin did not show any cytotoxic activity on healthy cell line used as a control, it exhibited significant antiproliferative activity on K562 chronic leukemia cells [42].

Besides the anticancer potential of levofloxacin that been reported, it has supportive effects for some...
complications in patients with receiving chemotherapy. It has been observed that febrile neutropenia, a complication that often develops in patients receiving cancer chemotherapy, reduced fever and supported neutrophil recovery after the treatment of levofloxacin [43, 44]. In addition to levofloxacin effectiveness for some side effects after chemotherapy, our results suggested that levofloxacin might be safe to use with it’s own impact on cancer cells while it was working for it’s own antibiotic pathway. In our study, even though the single levofloxacin toxicity was found lower on tongue type OSCC, the results were pointed out the apoptosis thorough it’s own toxicity. It was thought that the wide range of concentrations for their combinations is needed for future aspects with further analyses. The less toxic alternatives but still effective apoptosis capacity of levofloxacin as antibiotic combinations with less concentrated cisplatin combinations might minimize the side effects of strong drugs unless blocking the effect of cisplatin. We have suggested that the area need more outcomes based on the interactions after our preliminary outcomes and limitations, to enlighten the ambivalent mechanism for levofloxacin and cisplatin combination. On the other hand, it has been thought that single levofloxacin might be safe to use for patients independent of combinations, in addition to it’s bactericidal impact it has also toxic impact on cancer cells alone.

According to our study, the results for the treatment with levofloxacin and cisplatin combination were found controversial. Moreover, it has been found that the treatment with only levofloxacin may demonstrate the feature of potential safe use for the patients. On the other hand, the combination treatment has a less toxic effect than single cisplatin treatment but still a successful apoptosis rate on SCC-15 cells. Our preliminary study results have pointed out a question that single levofloxacin comes up with safe usage potential for OSCC patients who are suffering an infection even for reducing the possible side effects of cisplatin but the actual question is that how about using levofloxacin with cisplatin at the same time, even if the combination was triggered apoptosis?

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Declarations

Conflict of interest There is no conflict of interest about this article to declare.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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