Levofloxacin Might be Safe to use for OSCC Patients

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Abstract

Purpose

Since oral squamous cell carcinoma (OSCC) patients are exhausted against the powerful chemotherapies and radiotherapies after surgeries, therefore most of the studies still look for less toxic but effective alternatives with new ideas such as antibiotic combinations.

Methods

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 on a healthy cell line (MRC-5).

Results

24h treatment of 50 mM single levofloxacin, 50 mM single cisplatin and 50 mM levofloxacin-cisplatin combination resulted in cell 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% and 16.21% respectively on SCC-15. Also MRC-5 were showed the lower toxicity than cancer cells via apoptosis.

Conclusion

Levofloxacin-cisplatin combination results have also ended up apoptotic results with less toxicity for cells than single cisplatin treatment. Therefore, our apoptotic findings suggest that the different dosage combinations with levofloxacin and cisplatin are necessary to understand the interaction for the treatment of tongue squamous cell carcinoma.

Introduction

Oral cavity cancers have been following the top-ten common cancer in the world, and it includes epidermoid carcinomas, salivary gland carcinomas, lymphomas, sarcomas, and melanomas (Pan et al. 2014). 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 (Miguelanez-Medran et a. 2019). Some life choices that people can choose to do or not 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 it's around with a lot of people who are not choosing the 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 is deriving from epithelial squamous, including parts such as the tongue, buccal surface, floor of the mouth, soft and hard palates (Chaturvedi 2013; Pires 2013). The tongue type OSCC risk for young adult patients is increasing rapidly and will be increased more in the future (Paderno et al. 2018). 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 (Selvamani et al. 2015). 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 bad prognosis (Sultana et al. 2014). 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 (Sultana 2014; Almangush 2020). In the early stages of the disease, surgical excisions might provide a good survival rates. On the other hand, in addition to decreasing level of survival in advanced-stage tumors, using radiotherapy and chemotherapy including more and prolonged side effects might be exhausted for patients (Kim et al. 2020). Cisplatin is one of the best effective chemotherapeutic agents and widely used in many cancer treatments including the OSCC (Dasari et al. 2020), but still controversial in OSCC treatment because of acquired drug resistance and side effects in general cancer treatments (Yoshikawa et al. 2015).

Nevertheless, fighting with cancer make the body vulnerable to infections either with side effects or immunity changes. Using the right 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 the ancient times and still on the table by 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 (Bayat Mokhtari et al. 2017) to get repress or even lower 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 to investigate their antiproliferative and apoptotic effects (Dong 2019; Yedav 2019). 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 (Yedav 2019; Dalhoff 2003).

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 (Oliphant 2002). 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 (Yedav et al. 2019). Levofloxacin is a fluoroquinolone antibiotic that has the area to treat upper airway related diseases and for many bacterial infections in the body (Izadi et al. 2019). 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 (Fish et al. 1997). There are also studies that investigating the potential anticancer activity of levofloxacin when administered with different techniques (Gouvea 2012; Kljun 2013; Yoshimura 1996; Mondal 2004; Yu 2016; Song 2016). Yu et al. showed that levofloxacin selectively inhibits cancer cell proliferation on 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 (Yu et al. 2016). In another study, anti-proliferative and pro-apoptotic activities of levofloxacin were demonstrated using lung cancer cell lines and xenograft lung tumor model (Song et al. 2016). On the other hand, there are studies that showing levofloxacin derivatives also exhibit anticancer activity on different types of cancer (Korolyoc 2010; Sun 2013). 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 (Sun et al. 2013).

Another dilemma and question to find in usage of antibiotics in cancer patients is drug interactions. We encounter a variety of infectious pathogens, often atypical for healthy patients, in cancer patients. These infections are experienced more resistantly by cancer patients and their treatment requires wide spectrum antibiotics, mostly a combination of a couple. 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.

Prescribing antibiotics with several doubts for cancer patients is needed to be enlightened eventually in the line of RDU 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 increasing 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 targeted 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: 63087053) and MRC-5 (CCL-171) normal lung cell line (LOT:63405646) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). For the SCC-15 cells, 1:1 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 37ºC and 5% CO_2 for both cells. We did not add any other antibiotic (penicillin-streptomycin, gentamicin, et cetera) in our mediums to preserve the levofloxacin effect.

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 Technologies (Grand Island, MO, USA). Trypsin-Ethylenediaminetetraacetic-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 cm^2 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 x 10^5 cells were added in and then plates were put in an incubator. Following 24 h incubation step, cells were treated with determined dosages of levoflaxacin and cisplatin in between 5-100 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 ql 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 3,5 – 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 x 10^6 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 from wells and prepared for caspase-3 experiment. Treated cells were taken into the 2 ml tubes. Centrifugation step was made for 5 min at 800 rpm. Only the cell pellets were kept in the tubes and 100 ql cell lysis buffer was added on. Following the incubation step on the ice for 10 minutes, tubes were centrifuged again for 1 min at 10,000 rpm this time. Cell liquid phases were taken into the new tubes and 100 microliters cell lysis buffer added on. Each sample was divided in half and transferred to the two wells in a 96-well plate and 50 microliters of reaction buffer were added 1 to 1 over 50 microliter protein sample. Caspase-3 colorimetric substrate was added at 5 microliters per well. It was left at 37 ° C and 5% CO_2 for 2 hours, which are the normal incubation conditions. Measurement of absorbance values was carried out at 405 nm. To normalize the amount of protein, and the caspase-3 results, bicinchoninic acid (BCA) kit was studied for each well. Determining the caspase-3 increase based on the formation of the chromophore p-nitroaniline (p-NA) by cleavage from the labeled substrate DEVD-pNA, the apoptosis-induced of p-NA absorbance is compared with an unstimulated control absorbance.

Determining the apoptotic activity with Annexin V-FITC assay

Apoptotic activities of the cells were determined by measuring the DNA content of each cells on a fluorescence activated FITC-conjugated Annexin V-lectin. First, 0,1 x 10^6 cells were seeded in 6-well plates for each well, then every cell group treated with different drug doses except control wells. Following the incubation period, cell were centrifuged and supernatants were remowed. The binding buffers were added by homogenization way. 5 microliter FICT-Annexin V and same amount of PI reagents were added on cells. Following the vortex step, cells were stayed at 20–25□C and kept from light for 15 min. Results were measured in a flow cytometry device.

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 informations were 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

Levofloxacin-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 times 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 because of the clinical usage. After each drug and their combinations were administered for the MRC-5 cells as a healthy control, the results were toxic in these non-cancer cells on 48h and 72h incubation periods. When the time was increased the toxicity has become more effective for all cells. Considering this, for 24h 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 24h 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 the lowest toxicity with the 90% rate of on the SCC-15 cell viability. When their combination tested in 24 h, results were showed that cell viability was increased 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 than single cisplatin usage even though single levofloxacin has 10% of toxicity. Also, combination results have less cytotoxic effect on MRC-5 than SCC-15 cells (Fig. 2). Looking into with the toxicity ankle, after the drug interaction, toxicities 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 that no any 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 were 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 hours on MRC-5 cells.

Apoptotic activity

Apoptotic activity of SCC-15 and MRC-5 were studied by Muse annexin analyser device. The SCC-15 and MRC-5 cells were treated with three different groups on 24 h time period; 50mM doses of levofloxacin (Levo), 50 mM cisplatin (Cis) and combination of these two drugs (Levo-Cis) This method based on detection of the phosphotydilserine on the cell surface of the apoptotic cells after treatments for apoptotic sign. For each two cell group for all treatment; live cell, dead cell, early and the late apoptosis cells were identified. 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). In another words, single levofloxacin results were still showing the safe usage potential for patients however levofloxacin and cisplatin combination was marked with controversial. On the other ankle, the combination has less toxic effect than single cisplatin but still successful apoptosis rate on SCC-15 cells.

Discussion

Oral squamous cell carcinoma (OSCC) is the most frequent type of oral cancer, which accounts for 95% of all oral cavity and oropharyngeal cancers (Villagómez-Ortíz et al. 2016) its incidence increasing every year (Villagómez-Ortíz 2016; Blatt 2017). The clinical course of the disease and the treatment method to be applied vary depending on the primary tumor location, size and stage (Ettinger et al. 2019). Adjuvant treatment methods such as chemotherapy and radiotherapy together with surgery constitute the basic treatment standards (Ettinger 2019; Omura 2014). Even though advances in the treatment options, the rapid proliferation of cancer cells and high metastasis potential of the disease make the treatment process difficult and reduces the 5-year survival rate. Moreover, the ability of cancer cells to develop drug resistance and the side effects of chemotherapeutic agents and drug efficiencies are the main problems encountered in the treatment of the disease. Cancer patients are also having the less defence mechanisms for fighting with the infections especially after their chemotherapy and radiotherapy sessions. Prescribing the right antibiotics for cancer patients is important not only because for patients vulnerable immune systems but also not knowing the antibiotic's impact on the cancer cells. In our study we have examined the capacity of single levofloxacin and it's combination with cisplatin for treatment alternatives. The results were showed that levofloxacin and cisplatin combination has the less toxic influence but still successful apoptotic outcome in contrast to single cisplatin caused itself. Their combination results were showed the small level of reducing the toxic activity and apoptosis rate than single cisplatin achieved itself but still triggered the apoptosis.

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 (Qi et al. 2019). In many studies, it has been determined that cisplatin showed anticancer activity in many solid tumors such as lung, ovary and breast cancer (Shi 2016; Al-Bahlani 2017; Kleih 2019). 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, ototoxicty 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 (Pezeshki et al. 2017). The most common side effects

of cisplatin are nausea and vomiting due to its toxicity on the gastrointestinal system (Qi 2019; Tsang 2019).

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 new options that may be beneficial (Bhatia et al. 2020). Particularly, antibiotics have been reported to have anticancer activities, similar to their effects on bacteria in treatment of bacterial infections (Bhattacharya et al. 2015). Levofloxacin, one of the antibiotics with anticancer activity, is a second-generation fluoroquinolone used in the treatment of upper respiratory tract-related infections. Its anticancer activity has begun to be demonstrated in many investigations. For instance, levofloxacin inhibited cell proliferation and improve apoptosis in lung cancer cells. And also inhibited mitochondrial electron chain function leading to reducing of ATP production (Song et al. 2016). In breast cancer cells, levofloxacin inhibited mitochondiral biogenesis via supression 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 (Yu et al. 2016). In another study, researchers evaluated the anticancer activities of 15 acidic drugs selected from 8 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 (AlKhalil et al. 2020).

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 with receiving cancer chemotherapy, reduced fever and supported neutrophil recovery after the treatment of levofloxacin (He 2015; Olson 2021). In addition to levofloxacin effectiveness for some side effects after chemotherapy, our results were 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 independently of combinations in addition to it’s bactericidal impact it has also toxic impact on cancer cells alone.

Our preliminary study results have pointed out a question that single levofloxacin come up safe usage potential for OSCC patients who are suffering an infection even for reducing the possible side effects of cisplatin but how about using levofloxacin with cisplatin at the same time even though the combination was triggered apoptosis?

Declarations

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

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Figures

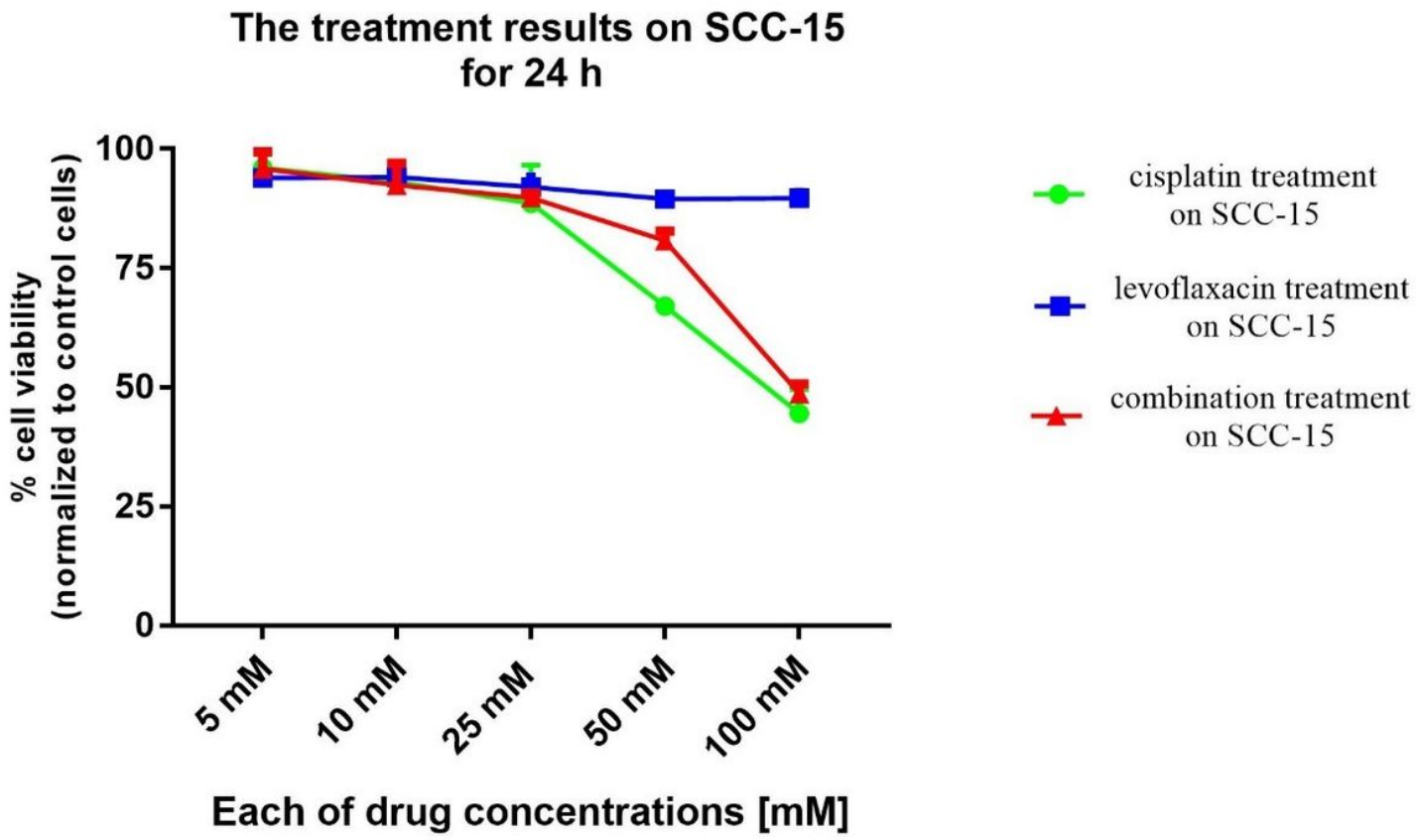

Figure 1

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. Each dose treatment results were repeated for three times. Dunnett's multiple comparasion test was performed in GraphPad PRISM v 7.04. Error bars represent for standart deviation.

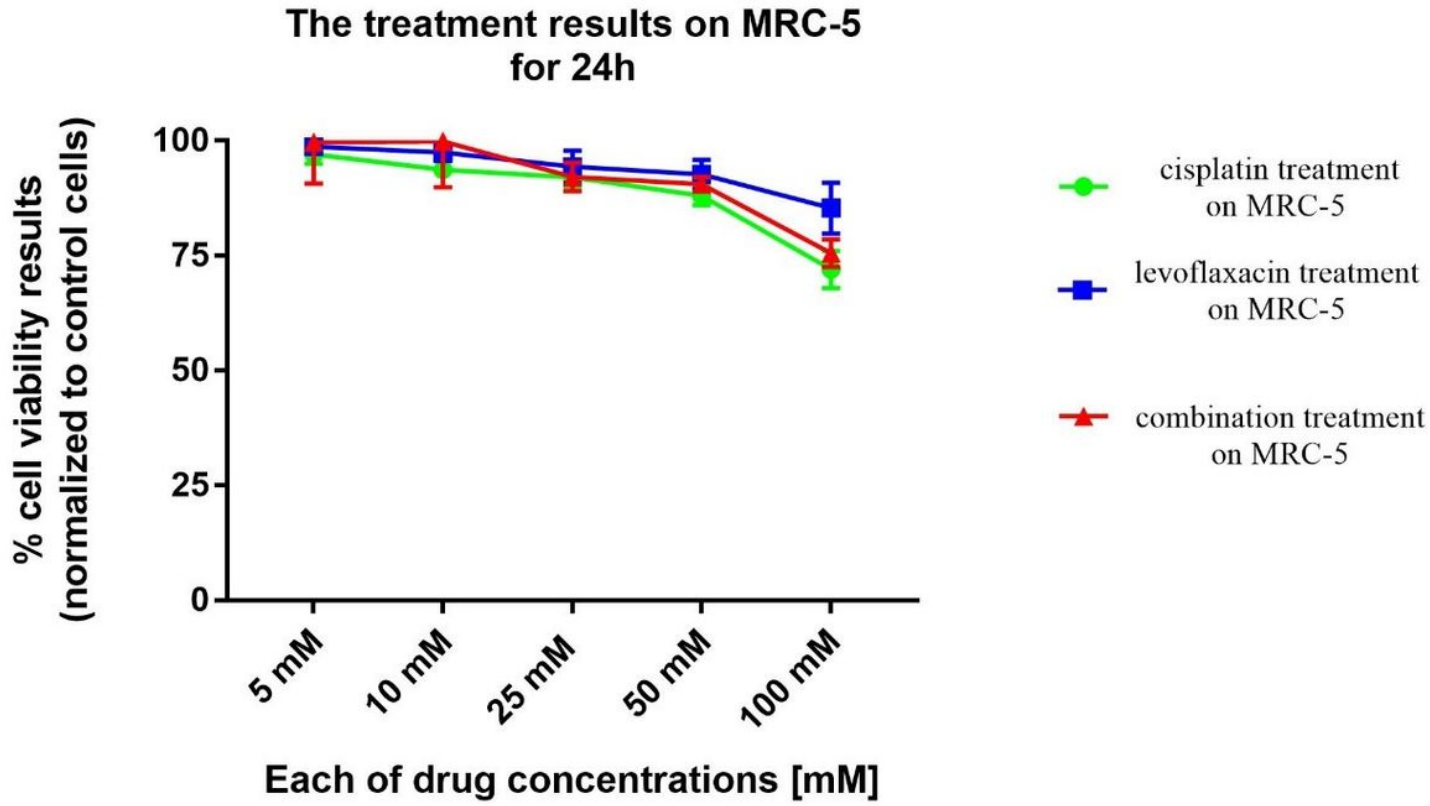

Figure 2

The cell viability for each drug and combinations on MRC-5 cells, that were represented on connecting line groups, each shape represents the one drug dose. Each dose treatment results were repeated for three times. Dunnett's multiple comparasion test was performed in GraphPad PRISM v 7.04. Error bars represent for standart deviation.

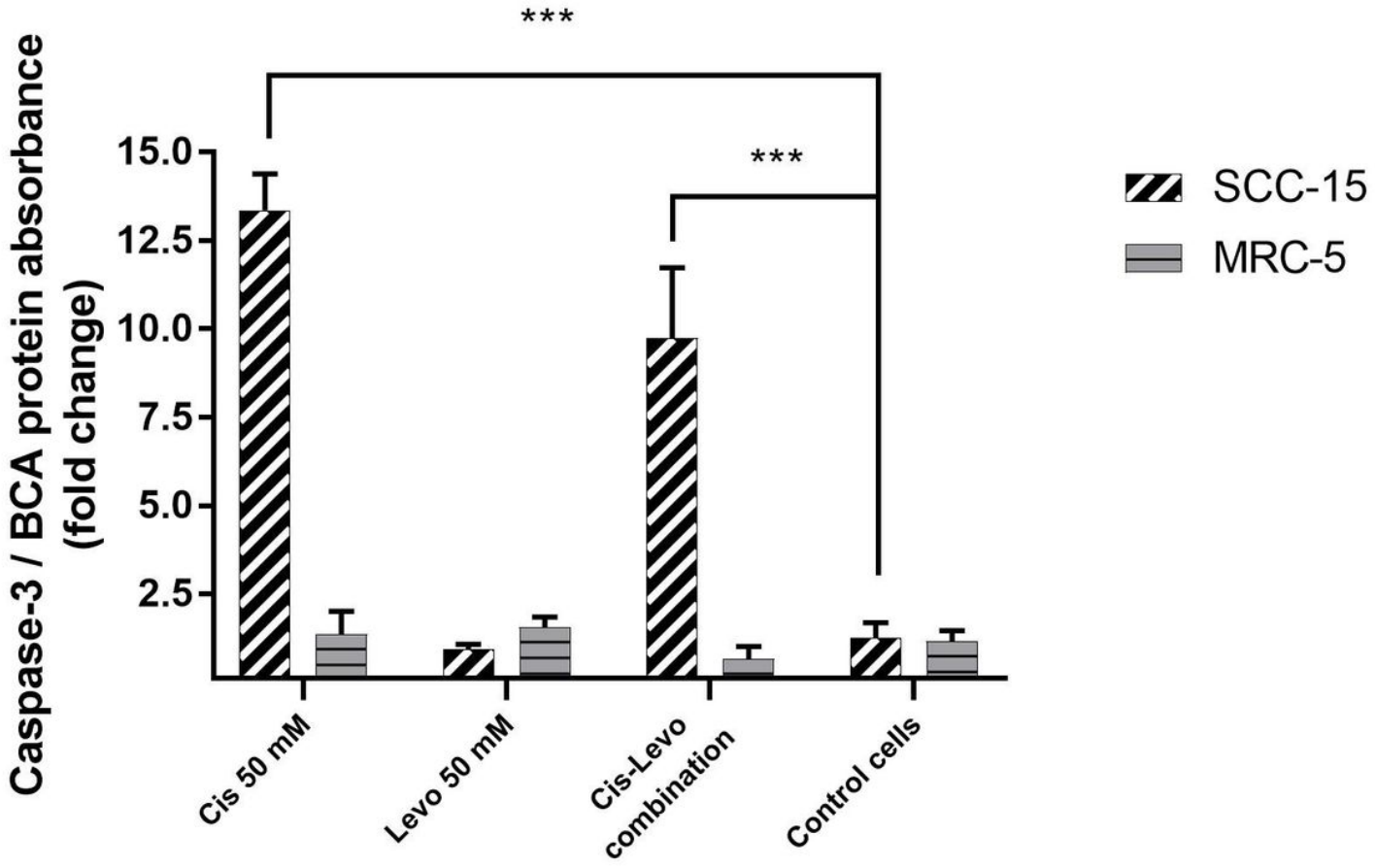

Figure 3

The caspase-3/BCA activity, that was represented on bar graph, and bars represent the three replicative results. Dunnett's multiple comparasion test was performed in GraphPad PRISM v 7.04. Three asteriks (***) sign for $p \leq 0.001$ (versus control cells). Error bars represent the standart deviation.

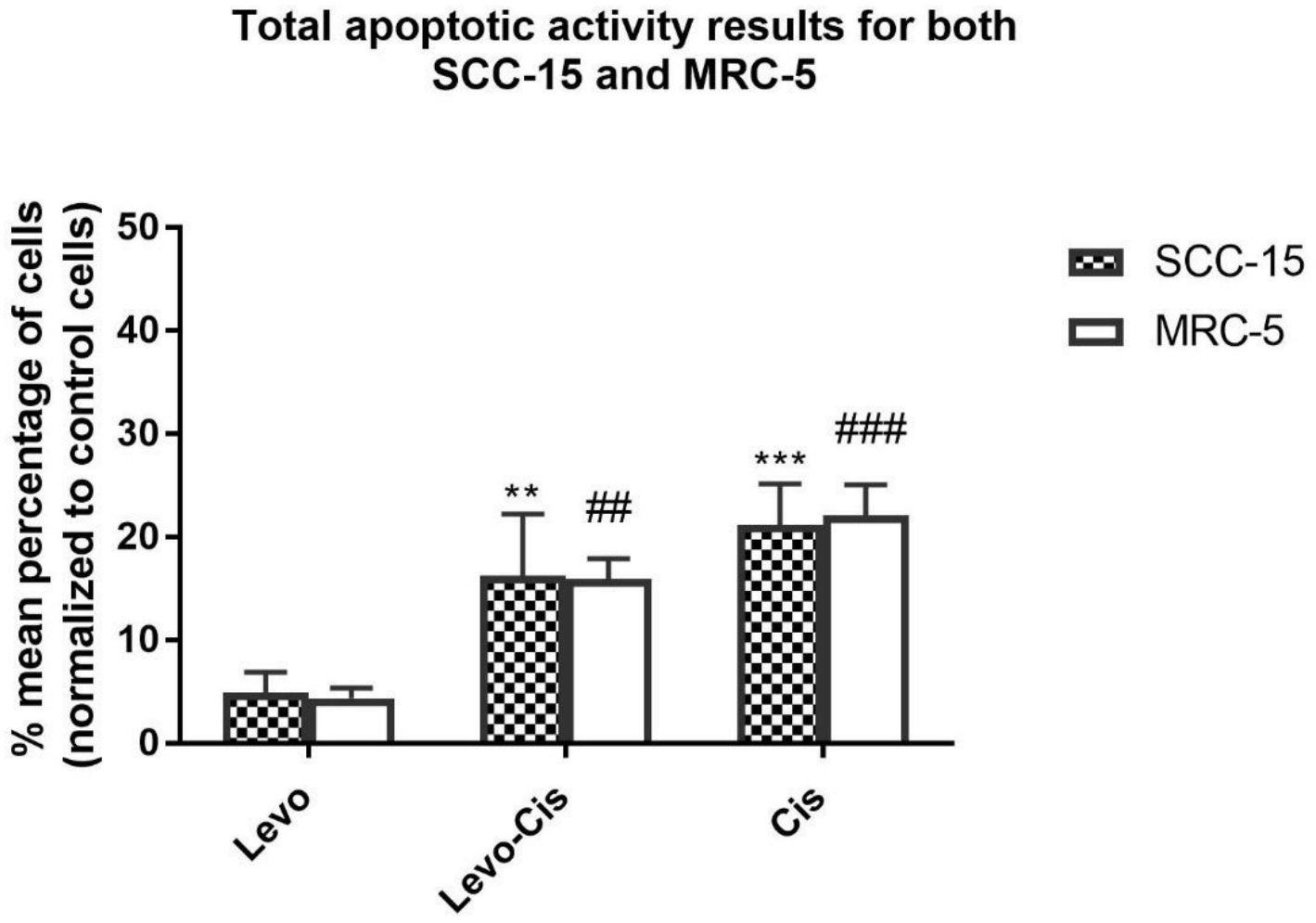

Figure 4

The percentages of total apoptotic results were represented on bar graph for each drug treatment. Each bar represents the median for three replicative results. Tukey's multiple comparasion test was performed in GraphPad PRISM v 7.04. Two asteriks (**) sign for $p \leq 0.01$, and three asteriks (***) sign for $p \leq 0.001$ compared to only levo treatment on SCC-15. Two sharps (##) sign for $p \leq 0.01$, and three sharps (###) sign for $p \leq 0.001$ compared to only levo treatment on MRC-5. Error bars represent the standart deviation.