Original Article

Cytotoxicity effect of ozone in oral squamous cell carcinoma: An in vitro study

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, approximately 90-95% of the total malignancy in the oral cavity. Eradicating cancer cells with radiation and chemotherapy have been concerned as cancer treatment methods, that tend to reduce immune system levels and activity. Ozone therapy has been developed to treat cancer without affecting the immune system. The objective of the study is to determine the cytotoxic effect of ozone and ozone combined with cisplatin against OSCC CAL-27 culture cells. OSCC CAL27 cells were cultured in 96 well plates. The MTS method is used as a cytotoxic test with DMEM as a media control, DMEM and cells as a negative control, cisplatin as a positive control, ozone dissolved in the media, and a combination of ozone and cisplatin as a treatment group whose dose is determined (500, 750, 1000, 1125, 1500, 3125, 3750 mg/mL). The double experiment was assessed with 4 replicates in each experiment. The corrected absorbance value was used to assess the effect of cytotoxicity. ANOVA statistical tests in experiments 1 and 2 showed a p-value <0.01 single-dose ozone, and a p-value <0.05 in a combination of ozone and cisplatin. The Pearson correlation test shows a strong correlation (0.711) in the first experiment and a very strong correlation (0.812) in the second experiment. Ozone has a cytotoxic effect on OSCC CAL-27 culture and ozone combined with cisplatin has a higher cytotoxic effect than single-dose ozone, so it can be considered a cancer adjuvant therapy.

Keywords: Oral squamous cell carcinoma, Cytotoxicity, Ozone, Cisplatin, Absorbance

Introduction

The case of oral malignant tumors in Indonesia accounts for 3-4% of all malignancies with various tumor etiologies (multifactorial). The mortality rate is 2-3% of all deaths due to malignancy. The national prevalence of oral cancer in Indonesia in 2007 was 0.2% [1]. Squamous cell carcinoma (SCC) is cancer originating from the epithelium tissue with a clustered cell structure and is capable of infiltrating through the bloodstream and lymphatics that spread throughout the body [2, 3]. Oral squamous cell carcinoma (OSCC) is a type of cancer that most often occurs in the oral cavity with a total malignancy of 90-95%. The locations of oral squamous cell carcinoma are usually on the tongue (ventral and lateral), lips, the floor of the mouth, buccal mucosa, and retromolar area [4-6]. The wide range of clinical appearances during its early stage can cause delayed diagnosis and treatment [7]. Tongue OSCC ranges from 25 to 50% of all malignant cancers in the mouth and has a poor prognosis if metastases have occurred to other areas (neck and cervical) [8].

The methods and means of cancer treatment depend on the type of cancer itself which can currently be identified by focusing on the eradication of cancer cells by radiation and chemotherapy methods. Several studies have shown that both methods tend to reduce levels and activity of the immune system [9, 10]. Cisplatin, the metal-based chemotherapy used for the treatment of patients with various types of malignancies, including squamous cell carcinoma [11, 12]. One of the treatments for

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cancer that can be done without affecting the immune system is ozone therapy [13]. Ozone, a gas that was discovered in the mid-19th century, is a molecule consisting of three oxygen atoms. It is also an unstable dynamic structure due to mesomeric conditions. Ozone gas is colorless, has a strong odor, and is explosive in liquid or solid form. The half-life of ozone is 40 minutes at 20 ºC and about 140 minutes at 0 ºC. The main function of ozone is to protect humans from the harmful effects of UV radiation. Ozone is formed less than 20µg/m3 from the earth's surface at concentrations suitable for life. Although ozone has harmful effects, some studies believe that ozone also has a therapeutic effect [14-16]. Ozone at the right dose provides a cytotoxic effect on cancer cells but did not damage normal cells and is no-carcinogenic.

Research on the effect of ozone on cancer cell death in cell culture, states that ozone can induce cell death in certain doses. The cell culture method is one way in vitro research that can be used. In the field of oncology, ozone is used in cancer cells for peroxide intolerance. Ozone produces reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which is why the mechanism of ozone against cancer cells is cell damage caused by ROS [17]. Another mechanism is based on the inability of cancer cells to compensate for the oxygen burst of ozone compared to healthy cells, leading to cancer cell death [11]. Ozone, through its anti-inflammatory effect, suppresses the nuclear factor kappa B (NFkB), the most important factor involved in transcriptional processes in several inflammatory processes and cancer metabolism [18]. Inhibition of NFkB activation will trigger the activation of cyclin D. Inhibition of cyclin activation causes no phosphorylation of cancer proteins, so the cells will not enter the synthesis phase (DNA synthesis) [19].

The role of ozone in cancer treatment has been investigated, but there are relatively few studies on the benefits of ozone against cancer. In 1980, laboratory studies at the University of Washington found that ozone inhibited the growth of lung, breast, and uterine cancer cells at specific doses while healthy tissue was not damaged by ozone. Radiation and oncology studies have shown that ozone therapy can increase the oxygenation of hypoxic tumors. In 2004, The University of Oxford reported that the Spanish cancer research center conducted a human study using ozone involving 19 patients with head-neck malignant tumors and the result was that patients treated with ozone had a longer survival rate than those receiving chemotherapy. Several studies have also reported that ozone can improve the side effects of cancer therapy such as radiation injuries and the side effects of cisplatin chemotherapy and other drugs [13, 18-21]. Based on the aforementioned background, the authors are interested in researching the cytotoxic effect of ozone on OSCC in cell culture.

Materials and Methods

Materials and ethical issues

The cell samples used were CAL27 cell cultures, then treated with a single dose of ozone and a combination of cisplatin-ozone. CAL27 is a cell line of the human tongue's squamous carcinoma purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The research sample was divided into 9 groups with 4 repetitions, included negative control, positive control, and 7 groups dose samples. This research was conducted at the Central Laboratory of Padjadjaran University. Ethical permission has been obtained from the Research Ethics Committee of Padjadjaran University No. 1072/UN6.KEP/EC/2019.

The ozone used in this study was obtained from a medical ozone generator machine from the Center for Plasma Research, Diponegoro University, Semarang, which produced a controlled range of gas flow and ozone concentration. Medical ozone at several different doses **(Table 1)** was exposed to culture media for preliminary studies.

Table 1. Dissolved ozone dosage regulation

Adjust	Ozone Concentration (mg/l)		Capacity (Concentration x Flow) (mg/min)	Time (min)	Dosage (Capacity x Time) (mg)	Dissolved Dosage (Dosage ÷ vol) (mg/ml)	Label
Flow	0.9 l/ min	1.5 l/ min					
15		25	37.5	1	37.5	62.5	A
20		50	75	1	75	125	B
25		100	150	1	150	250	C
35		150	225	1	225	375	D
50		200	300	1	300	500	E
80	500		450	1	450	750	F
50		200	300	2	600	1000	G
35		150	225	3	675	1125	H
80	500		450	2	900	1500	I
50		200	300	4	1200	2000	J
80	500		450	3	1350	2250	K
85		250	375	5	1875	3125	L
80	500		450	5	2250	3750	M

Laboratory studies

The workflow in this study was to prepare a complete Dulbecco's Modified Eagle's Medium (DMEM) liquid culture medium (which contains 10% Fetal Bovine Serum (FBS) and antibiotics Penicillin-Streptomycin). The positive control used in this test was 53,48 ug/mL cisplatin. The cisplatin dosage experiment has been carried out before. The working solution used for the anti-proliferation assay was PrestoBlue ™ Cell Viability Reagent. Cells that had been at least 70% confluent were transferred to the dish, then the cells were rinsed 2x with 5 mL PBS. 1 mL of Trypsin-EDTA solution was added, then incubated for 5 minutes so that the cell layer was dispersed. The cells were moved into the tube containing the media. Centrifugation of cells was done at 3,000 rpm for 5 minutes. The supernatant was removed, then the pellets were dissolved into the tube containing the media. Cell culture was established into 96 well plates, then incubated for 72 hours (or until min. 70% confluent cells) at 37 ° C and 5% CO2 gas.

Seven 5 mL tubes were prepared, then each microtube was labeled with the appropriate dose concentration, namely: Ozone 500 mg/mL, ozone 750 mg/mL, ozone 1000 mg/mL, ozone

1125 mg/mL, ozone 1500 mg/mL, ozone 3125 mg/mL, ozone 3750 mg/mL. The dose was obtained from a preliminary test. The 96 well plates containing cells from the incubator were removed. The plates were labeled to note which rows were to be treated by the standard and which rows were to be sampled. Then the media from each well was removed. 100 µL of each single dose ozone sample solution, cisplatin combination ozone, and cisplatin positive control were transferred into each of the wells of the 96 cell-filled well plates using micropipettes. Then, they have incubated again for 48 hours. To find out the effects of ozone cytotoxic when added with the chemotherapy agent, cisplatin, ozone in the above doses were added with cisplatin in a ratio of 50:50.

The media of each well was removed. Nine mL of media was prepared on the falcon added by 1 mL of "PrestoBlue™ Cell Viability Reagent" (10 µl reagent for 90 µl media), then 100 µl of the solution mixture was poured into each good microplate then incubated for 1-2 hours until there was a change of color. Next, the absorbance at a wavelength of 570 nm (reference: 600 nm) was measured using a multimode reader. Treatment repetitions were carried out 4 times in 2 experiments.

Statistical analysis

To test the cytotoxic effect of ozone and ozone combine cisplatin on the growth of CAL-27 OSCC cell culture and compare it with controls, a one-way ANOVA ($p<0.01$) and ($p<0.05$) post hoc Bonferroni statistical test was performed. To test the association of the cytotoxic effect of the combination of ozone with cisplatin and ozone without the combination of cisplatin on the growth of cell culture OSCC CAL-27, a Pearson correlation test was performed.

Results and Discussion

Results

A preliminary study had been conducted to determine the range of cytotoxic effects of ozone on cells at certain doses carried out in this study. The dose-effect was evaluated using the quantitative MTS test. The result of the mean value of corrected absorbance is presented in **Figure 1**. The graph shows that at a starting dose of 500 mgmL, ozone has a significant effect on cell absorbance. The absorbance continues to decrease until a dose of 1500 mgmL and remains relatively constant thereafter. The mean values for the negative controls: (0.4986 + 0.4675)/2 = 0. 48305; IC-50 target: 0.48305/2 = 0.2415; and the IC-50 value in accordance with the graph y = -0.0002x + 0.5625, which is 1605 mg/mL.

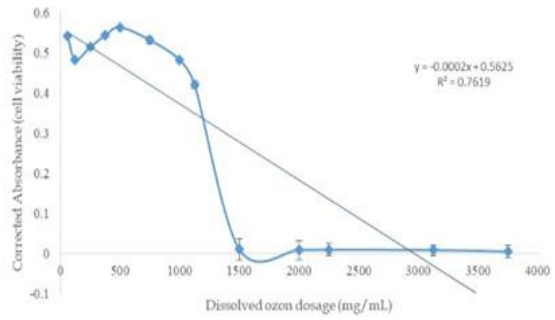


Figure 1. Comparison of ozone dose to corrected absorbance

The comparison between the absorbance value in the preliminary study, the first trial, and the second trial was presented in **Figure 2**. In the first experiment (red), the IC-50 target was obtained which is 0.2493 absorbance, and the IC-50 value was following the graph y = -0.0001x + 0.356, which is 772.75 mg/mL. Whereas in the second experiment (green), the IC-50 target was 0.2493 absorbance, and the IC-50 value was following the graph y = -0.0006x + 0.6308, which was 529.1 mg/mL. Thus, it can be concluded that the second experiment has a better IC-50 result than the first experiment.

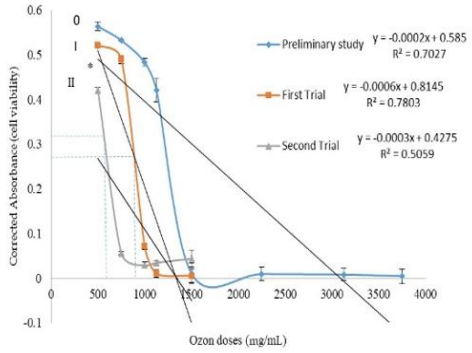
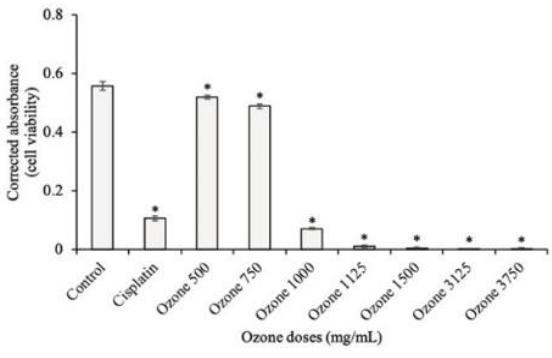
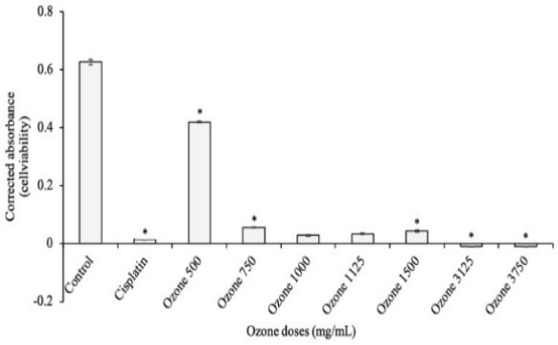


Figure 2. The comparison between the absorbance value in the preliminary study, the 1st trial and the 2nd trial

The ANOVA statistical test shows that ozone increased cytotoxic effect in CAL-27 ($p<0.01$). Based on the results of Bonferroni's multiple comparisons analysis, as shown in the appendix, significant differences were seen between each dose level compared with the negative control. The doses of 1000 mg/mL and 1125 mg/mL in the second trial had a cytotoxic effect that was relatively the same as the positive control (cisplatin). The statistical test results above are summarized in **Figure 3**.



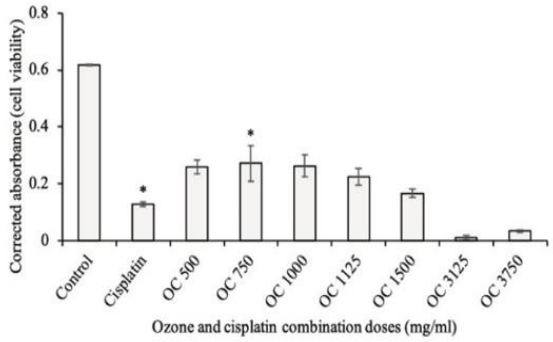
a)



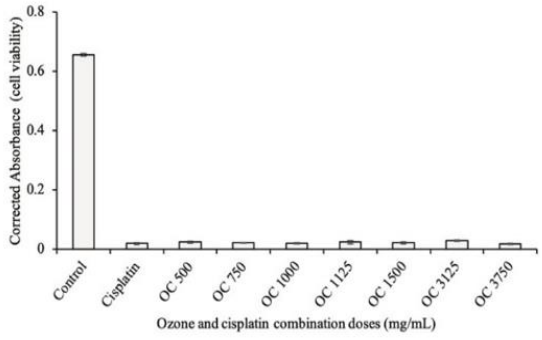
b)

Figure 3. Cytotoxic effect of ozone on CAL-27 cells compared to the control. a) (*p <0.01) In the 1st trial; b) (*p <0.01) In the 2nd trial.

When ozone combined with cisplatin, the ANOVA statistical test showed the value of p= 0.000, p <0.05, which means that there was a significant difference. Based on the results of Bonferroni's multiple comparisons analysis, as shown in the appendix, significant differences were seen between each dose level compared with the negative control. The dose of 750 mg/mL in the first trial had different cytotoxic effects compared to the positive control (cisplatin). Whereas in the second trial, there was no significant difference between each dose and control cisplatin. The statistical test results were summarized in **Figure 4**.



a)

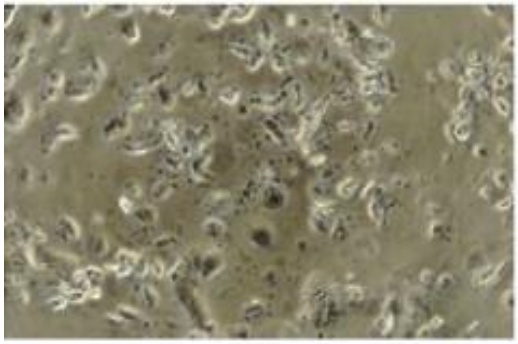


b)

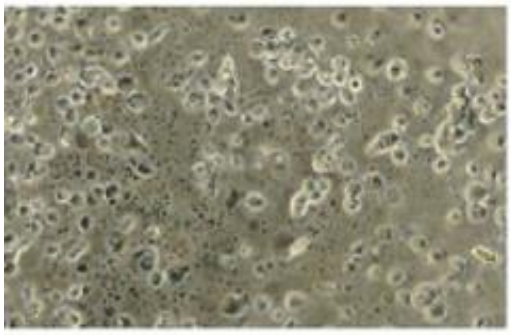
Figure 4. Cytotoxic effect of the combination of ozone and cisplatin (OC) on CAL-27 cells compared to the control. a) (*p <0.05) In the 1st trial; b) (*p <0.05) In the 2nd trial.

The statistical analysis showed that there was a correlation of cytotoxic effect between the combination of ozone cisplatin and ozone without the combination on CAL-27 cells. The Pearson correlation value in the first experiment was 0.711 revealed that it has a strong correlation, while the Pearson correlation value in the second experiment was 0.812 which means it has a very strong correlation. Thus, it can be concluded that the addition of ozone to cisplatin produces a higher cytotoxic effect than ozone without the combination of cisplatin. **Figure 5** showed the microscopic examination of the three groups, they are the control cells, cisplatin-treated cells, and ozone treated-cells.

a)



b)



c)

Figure 5. a) The control cells; b) Cisplatin-treated cells; c) Ozone-treated cells (dose 1000 mg/mL)

Discussion

The results of this study indicated that ozone has a cytotoxic effect on CAL-27 cells at a certain dose which follows the increase in dose. Medical ozone in this study was able to provide cytotoxic effects starting from dissolved doses of 500 mg/mL up to 3750 mg/mL. This can be seen from the results of the corrected absorbance MTS which was measured after 48 hours after the cells were given treatment. The absorbance value is a quantitative parameter to evaluate cell viability. Cell viability is related to the activity of the oxidoreductase enzyme [13]. The

oxidoreductase enzyme functions as a catalyst for reactions of oxidation and reduction, which transfers electrons, hydrogen, and oxygen.

The cell viability listed in this study did not correlate closely with the morphological features of the cells. CAL-27 cells are epithelial cells taken from the tongue tissue of human squamous cell carcinoma. The epithelial cells are polygonal with highly granulated cytoplasm as shown in the negative control morphology (media and cells). In positive control using cisplatin, it was seen that the cytoplasm began to shrink, round, and apoptosis. At doses of 3125 mg/mL and 3750 mg/ml, the cell morphology was similar to that of negative controls, although the MTS test showed the lowest cell viability results. This could occur due to the influence of ozone at this dose which affected mitochondrial activity without damaging the overall cell anatomy so that the MTS test did not find mitochondrial metabolic activity.

Medical ozone in this study was a mixture of ozone-oxygen that is exposed to the culture medium and causes the formation of ozone-bioorganic compounds (ozonides) interaction products. These products can damage cell membranes and inhibit oxidoreductase enzyme activity. It can be seen that there was a decrease in cell viability by 93.3% in the first experiment and 67% in the second experiment starting at a dose of 500 mg/mL. Alyasova, *et al*. (2016) showed that the viability of cancer cells was greater than normal cells (4.54% in normal cells and 15.7% in cancer cells) with ozone-oxygen treatment. This quantitative difference may explain that there is greater antioxidant protection in cancer cells [13].

Several other studies, including Poma, *et al*. (2017), on the effect of ozone on adenocarcinoma cancer cell cultures and normal fibroblast cells showed that at a dose of 120 ppb, ozone had a greater genotoxic effect on cancer cells than normal cells [20]. This shows that ozone does not cause oxidative stress in normal cells, so it is relatively safe to use in cancer therapy. Another study on the effect of ozone on normal human bronchial epithelial cell culture by Gabrielson (2014), was done by measuring the release of the enzyme lactate dehydrogenase (LDH). The study showed that ozone reduces the ability of cells to replicate at low levels (≤ 1 ppm), and does not cause cytolysis. Replication has an important impact on the ability of cells and tissues to repair premature damage [22]. Research by Kuroda, *et al*. (2015), on the antitumor effect of water dissolved ozone in vivo, showed that ozone has a selective antitumor effect on tumor cells and does not affect normal cells [17].

Ozone therapy is an alternative therapeutic method that is starting to be researched and developed. Several studies have shown that ozone has a proliferative inhibitory effect on cell cultures of lung cancer, breast cancer, uterine tumors [23, 24], cervical cancer [20], and liver cancer [13]. Medical ozone has the paradoxical effect of ozone, meaning that it acts as a stimulator of antioxidant defenses in humans, even though ozone is a direct cellular oxidant [25, 26]. The working mechanism of ozone biologically is acting as a trigger for oxidative stress on antioxidants and membrane polyunsaturated fatty acids (PUFA) in the lipid peroxidase process. This mechanism stimulates the production of secondary signals, which are hydrogen peroxide (H2O2) and alkenal (especially 4-hydroxynonenal, 4-HNE), both of which can activate nuclear factor- (erythroid-derived 2) like 2 (Nrf2). This results in antioxidant and anti-inflammatory effects based on the induction of superoxide dismutase, glutathione-peroxidase, heat shock protein (HSP-70), and heme oxygenase-1 (HO-1). Nrf2 as an ozone anti-inflammatory effect agent suppresses nuclear factor kappa B (NFKB).

The addition of cisplatin and ozone into culture media was able to provide a higher cytotoxic effect than ozone without the combination of cisplatin. The Pearson correlation statistical test showed that the p-value was 0.000 ($p < 0.01$), with the Pearson correlation value in the 1st trial of 0.711 which means it has a strong correlation, while the Pearson correlation value in the second experiment was 0.812 which means it has a very strong correlation. Based on these statistical results, the addition of ozone to cisplatin produces a higher cytotoxic effect than ozone without the combination of cisplatin.

Cisplatin and other chemotherapy agents have side effects which include damage to normal cells. Ozone has selective inhibition of cancer cell growth.22 A study on the addition of ozone to chemotherapy agents on the viability of hepatic cancer cell cultures (2016) showed that the ozone-doxorubicin combination was better than the combination of ozone without combination, and the combination of oxygen-doxorubicin [13]. Thus, the addition of ozone to chemotherapy agents is expected to reduce the aforementioned side effects, especially in the oral cavity. Ozone can bind tightly to tissues when it comes in contact with organic fluids such as saliva, plasma, urine, and lymphoid fluids. These interactions depend on the concentration of ozone and will react with PUFAs, antioxidants, cysteine, glutathione, albumin, carbohydrates, enzymes, DNA, and RNA. All of the above reagents act as electrons and can be oxidized together with the process of ozonation to form ROS and LOPs which are responsible as biochemical agents in regulating inflammation and tissue healing [27]. The study limitation was time-level cytotoxic effects were not performed.

Conclusion

Ozone has a cytotoxic effect on OSCC CAL-27 cultures. Ozone combined with cisplatin has a higher cytotoxic effect in CAL-27 OSCC cultures than single-dose ozone, so it can be considered to be an adjuvant cancer therapy. Further studies are required testing based on time levels.

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Ethics statement: None

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