The ultrastructural surface morphology of oral cancer cells and keratinocytes after exposure to chitosan

Fatimah¹, A S Sarsito² and Y S Wimardhani²*
Department of Oral Medicine, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia
*E-mail: yuniardini@ui.ac.id

Abstract. Low-molecular-weight chitosan (LMWC) has the same selective cytotoxic effects on oral cancer cells as cisplatin. The cell deaths caused by the anticancer characteristics of chitosan show that apoptosis is not the death pathway of the primary cells involved. The interactions between LMWC and the cells need to be explored. The objective of this study was to compare the ultrastructural morphology of oral Squamous Cell Carcinoma (SCC Ca)-922 and noncancer keratinocyte HaCaT cell lines after exposure to LMWC and cisplatin. The cells were treated with LMWC and cisplatin, and their ultrastructural morphology was analyzed using scanning electron micrographs. Features of early apoptosis, seen as the loss of microvilli, were detected in the LMWC-exposed Ca9-22 cells, and there was a material surrounding the cells. In contrast, the LMWC-exposed HaCaT cells showed no changes related to apoptosis. The results were the opposite when cisplatin was used. This study confirms that there are differences in the ultrastructural surface morphology of LMWC-exposed and cisplatin-exposed oral cancer cells and keratinocytes that could be correlated with their biological activity.

1. Introduction
Cancer is still one of the world’s major health problems, resulting in high mortality rates. In 2005, mortality due to cancer accounted for about 15% of all death cases. It is estimated that the mortality rate due to cancer will continue to increase to 20% by 2030 [1]. The cancer problem is also one of the most important topics in dentistry. Oral cancer is an important health problem in the United States and other parts of the world, as the incidence of oral and pharynx cancers reaches 30,000 cases each year. Oral cancer is defined as cancer originating from the entire mucosa of the oral cavity, tonsils, pharynx, and larynx [2]. A total of 91% of all oral cancers are oral squamous cell carcinoma (OSCC). This type of cancer ranks as the sixth-most common cancer in developed countries, affecting about 44,000 patients and causing 11,000 deaths each year in the United States [1]. The increasing incidence of OSCC in Asia has reached 80% of the total cases recorded worldwide. According to the WHO, the incidence of OSCC in southern Asia is as high as 25 cases/100,000 people [2]. The etiology of OSCC is very complex and involves several factors that increase the risk of malignancy [3].

The prognosis of patients with oral cancer is determined by the stage of the disease at the time the patients are diagnosed. The stage of the disease also determines the type of treatment a patient should receive. The high numbers of patients who present with advanced-stage cancer cause the prognosis to be less favorable and the survival rate of patients with oral cancer to be only about 50% in recent decades [4]. One treatment is chemotherapy. Currently, chemotherapy is given as an induction therapy in patients at advanced stages if the tumor tissue cannot be removed immediately. Chemotherapy is defined as a systemic cancer therapy in the form of direct intravenous injections, intravenous infusions, tablets, or
capsules that contain anticancer or cytotoxic drugs. These drugs disrupt the growth of the cancer cells and destroy them. Chemotherapy usually becomes a therapeutic modality for patients with advanced disease stages (III and IV) or in patients who have been treated with surgery and radiation but have recurrences. In principle, the chemotherapy agents will enter the blood circulation and destroy the cells that are actively dividing, as the drugs damage the molecules that regulate cell division or inhibit the chemical processes that occur when the cells are proliferating [5].

One of the drugs commonly used for oral cancer therapy is cisplatin. Cisplatin is a platinum-based chemotherapeutic compound that is commonly used to treat various cancers including sarcomas, some carcinomas, lymphomas, and tumor cells. Cisplatin works by attaching itself to the DNA of cancer cells, interfering with cell division, and preventing growth [6]. Chitosan is a positively charged polysaccharide that is a partial deacetylation product of chitin. Chitosan is widely present in shrimp and crab shells and has a randomly distributed structure of D-glucosamine and N-acetyl glucosamine groups [7]. The number of N-acetyl groups determines the degree of deacetylation of the chitosan. Chitosan is a widely used biomaterial because of its biocompatible, biodegradable, nontoxic, and adsorptive characteristics. Previous studies have shown that low-molecular-weight chitosan (LMWC) has selective cytotoxic effects on oral cancer cells compared to cisplatin [8]. Furthermore, observations of the cell death mechanisms of LMWC show that apoptosis is not the death pathway of the primary cells involved, so it remains to be investigated whether the other cell death mechanisms are the ones involved in the cytotoxic effects of LMWC on cancer cells [9].

The presence of microscopic changes in cells exposed to LMWC, compared to cisplatin, is very interesting and needs to be further investigated, as it is likely to provide an explanation of the interaction between LMWC and cancer cells [9]. To see more detailed changes in the cells, ultrastructural morphological analysis is required to gain information about what happens to these cells. This study is expected to add the knowledge of the anticancer activity of chitosan on cancer cells (especially oral cancer cells) and also to reveal the ultrastructural morphology of the cell surface of OSCC strains after exposure to LMWC and cisplatin.

2. Materials and Methods

This experimental laboratory test used Ca9-22 cells (JCRB0625, established at the Tokyo Medical and Dental School, Japan) and HaCaT cells (a nontumorigenic cell line derived from dermal keratinocytes) as the treatment and control cell cultures, respectively. The test materials in this study were LMWC (Sigma Cat. Not. 44886-9, Milwaukee, WI, USA) and cisplatin (Wako, Osaka, Japan). Before being used for the experiment, a solution of 2% chitosan (2 g chitosan in 100 ml of 1% acetic acid) and 10% cisplatin (a suspension stock made in dimethyl sulfoxide [DMSO]) were prepared. Scanning electron microscopy (SEM) analysis was conducted at the Laboratory of Biomedical Technology Research, Postgraduate Program, Universitas Indonesia.

2.1. Cell culture

The complete culture medium used was Dulbecco's Modified Eagle Medium (DMEM; Gibco Cat. No. 11965-092, Life Technologies, Carlsbad, CA, USA) with penicillin and streptomycin (Caisson Laboratories), containing 10 million U/L penicillin G sodium salt and 10,000 mg/l streptomycin sulfate plus 5% FBS. The culture medium was filtered using a 50 ml SartoriusMinisart single-use sterile syringe filter with a diameter of 0.2 μm. The cell stock was washed by adding 1 ml of the cell stock to 5 ml of the medium in a15-ml tube, which was then centrifuged at 2000 rpm for 5 minutes at 24 °C. Then, the cell pellet was resuspended by adding 3 ml of the complete medium. The cells were plated on a culture dish and then cultured in a CO2 incubator at 37 °C and 5% CO2 until 90% of the cells were confluent. A total of 1×10^6 cells were then inserted into the culture dish and incubated for 1 hour before chitosan exposure.
2.2. **LMWC Exposure**

After incubation for 1 hour, the treatment group was exposed to 800 μg/ml chitosan (IC50) and 8 μg/ml cisplatin (IC50) [8]. HEPES solution at a concentration of 200 μg/ml was added to the treatment group exposed to chitosan. Cells in this condition were then incubated for 24 hours.

2.3. **Cell Fixation**

After 24 hours of exposure, the medium was removed, and the samples were washed with 1XPBS. Fixation was performed before the SEM procedure by adding 2.5% glutaraldehyde for 30 minutes twice. Dehydration was performed using alcohol with a sequence of concentrations from 25% up to 100% for 5 minutes.

2.4. **SEM Analysis**

After the cells were fixed, a coverslip was placed on parafilm to be dried, preventing the attachment of the coverslip to the culture dish. After the sample was dry, vacuuming and gold-coating were performed with an Emitech for 6 hours with a pressure of $10^4$ Mbar/Pa.

3. **Results and Discussion**

3.1. **Results**

In this study, laboratory experiments were conducted to determine the anticancer effects of chitosan and cisplatin on Ca9-22 cells during a 24-hour exposure period. This study also examined the anticancer effects of chitosan and cisplatin against HaCaT cells. This study used samples of cultured cell lines in a CO2 incubator at conditions of 37°C and 5% CO2. Each cell type was grouped into two groups: the control group and the treatment group. The control group was a group of cells that was not exposed to chitosan or cisplatin, while the treatment group was further divided into two groups, with one group of cells was exposed to 2% LMWC in 1% acetic acid and one group exposed to cisplatin. The microscopic features of each group can be seen in Figure 1.

![Figure 1](image-url). The microscopic features of Ca9-22: (A) control cells; (B) cells exposed to 800 μg/ml chitosan; (C) cells exposed to 8 μg/ml cisplatin

This study used a SEM to examine the ultrastructural morphological features of control cells and those exposed to LMWC and cisplatin. Figure 2 shows the ultrastructural morphological changes of Ca9-22 cell surfaces after exposure to chitosan and cisplatin.
The effects of chitosan and cisplatin exposure on HaCaT cells were analyzed to compare the two compounds. SEM analysis was also used to look at the ultrastructural morphological changes in these cells. Figure 3 shows the features of treated HaCaT cells analyzed by SEM.

3.2 Discussion
Previous studies have shown that LMWC has cytotoxic effects on oral-cancer-strain cells [8]. This phenomenon likely occurs because there is proton transfer among the glucosamine units in LMWC, which can produce many binding sites on the cell membrane [10]. The required concentration of LMWC is 10 times greater than is needed of cisplatin, indicating that cisplatin is a superior anticancer material.
for oral cancer. However, LMWC demonstrates selective toxicity towards cancer cells and even has the opposite effect on noncancer cells [8]. This study is a pharmacodynamic test of LMWC. Furthermore, the selective toxicity characteristics possessed by LMWC make it a good prospect for development as an anticancer agent.

A previous study also showed that LMWC induced the proliferation of HaCaT cells at concentrations that were cytotoxic to the three types of OSCC cell lines [8]. This suggests that chitosan at low concentrations has selective toxicity to both cancer cells and noncancer cells. Another study that examined the effects of exposure to LMWC on dental pulp cells also showed mitogenic effects at concentrations below 0.1% [11]. The exact mechanisms underlying the differences in the effects on both types of cells are not fully understood. It is possible that in cell culture experiments, LMWC interacts with a growth factor that is present in the culture medium, thereby increasing the potential for the proliferation of the growth factor and protecting it from degradation. Conversely, at high concentrations, chitosan can absorb metal ions that are present in the culture medium, causing a decrease in the number of these ions that lead to cytotoxicity in the culture medium [12].

The exposure to LMWC or cisplatin at the IC50 value of Ca9-22 cells showed a different result than the controls. In the group exposed to LMWC, the cell surface became uneven compared to the control (Figure 2), as did the group that was exposed to cisplatin (Figure 2). Further research using a transmission electron microscope is needed to analyze the cell ultrastructural features after exposure to LMWC or cisplatin, to see clearly what is happening. This may be able to answer the question of the mechanism of the interaction between cells and chitosan.

The mechanism of chitosan action against cells is not widely known. Some previous studies linked it to the processes of cancer cell apoptosis and increased normal cell proliferation. Apoptosis, or programmed cell death, is an important phenomenon in cytotoxicity induced by antitumor agents. The apoptotic pathway begins with a variety of extracellular stimuli, such as DNA damage, heat shock, the loss of growth factors, and caspase activation. It has been reported that chitosan induces apoptosis in tumor cell cultures [13]. However, the other mechanism of death is necrosis. Necrosis is cell death resulting from acute cell damage or trauma (e.g. a lack of oxygen, extreme temperature changes, and mechanical injury); when such cell deaths occur uncontrollably, they can cause cellular damage, inflammatory responses, and potentially serious health problems [14]. A previous study showed that apoptosis is not the primary cell death mechanism caused by LMWC, compared with cisplatin. This was proven by an analysis using TUNEL, the expression of Caspase 3, 8, and 9, and the number of populations in the sub-G1 phase [8]. To understand this, this study aimed to analyze, using SEM, the ultrastructural morphology of Ca9-22 cell surfaces after exposure to LMWC and cisplatin exposure. The same treatment of HaCaT cells exposed to LMWC and cisplatin was used as a comparison. Based on micrographs of the ultrastructural morphology of Ca9-22 cell surfaces, it could be seen that cisplatin and chitosan had an effect on the morphological structure; the effect was different in the control group and treatment groups.

The ultrastructural morphologies prove that the workings of these two anticancer drugs are different in disabling different cancer cells. Figure 2B shows Ca9-22 cells after LMWC exposure, but in the figure, one cannot clearly see the ultrastructural morphology of the cell surfaces undergoing changes as a result of apoptosis mechanisms: the loss of microvilli, membrane blebbing, and apoptotic bodies [15]. The loss of microvilli was a feature seen in LMWC-exposed Ca9-22 cells in the first 24 hours (Figure 3B). When compared with the features seen in cisplatin-exposed Ca9-22 cells, the apoptosis process had begun to appear in a more advanced form, as the loss of microvilli and the presence of membrane blebbing were starting to be seen (Figure 2C). This is in contrast to previous studies that exposed chitosan nanoparticles to erythroleukemic cells that showed features of the necrosis process [16]. In this study, there was no visible damage to the integrity of the cell membranes exposed to LMWC and cisplatin, showing that there were different cell death mechanisms between LMWC and nanoparticulate chitosan toward cancer cells. The results of this study reinforce the results of previous studies showing that apoptotic markers were not found in cells exposed to LMWC for 24 hours [8]. This was due to the inception of apoptosis marker-marker expression by Ca9-22 cells exposed to LMWC, proven by an
The ultrastructural feature that also did not show apoptotic features. The opposite results were found in HaCaT cells, the noncancerous cells that were control cells. No HaCaT cells featured apoptosis or necrosis after 24 hours of LMWC exposure, but the opposite occurred after cisplatin exposure (Figure 3C). HaCaT cells exposed to cisplatin showed apoptotic bodies, which is a further phase of apoptosis. These results confirm the results of previous studies showing that exposure to cisplatin in HaCaT cells led to higher cell death than LMWC exposure [8].

4. Conclusion

This study concluded that there are differences in the ultrastructural surface morphology of LMWC-exposed and cisplatin-exposed oral cancer and keratinocyte cell lines. The ultrastructural surface morphology showed that the exposure of Ca9-22 cell lines to LMWC resulted only in very early features of apoptosis, while there was no effect of LMWC exposure on the HaCaT cell line. The exposure of the cells to cisplatin resulted in a more advanced ultra-structural surface morphology, indicating apoptosis.

References

[1] Chaturvedi A K, Anderson W F, Lortet-Tieulent J, Curado M P, Ferlay J, Franceschi S, Rosenberg P S, Bray F and Gillison M L 2013 Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J. Clin. Oncol. 31 4550-9.
[2] Ferlay J, Shin H R, Bray F, Forman D, Mathers C and Parkin D M 2010 Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int. J. Cancer. 127 2893-917.
[3] Scully C 2011 Oral cancer aetiopathogenesis; past, present and future aspects. Med. Oral. Patol. Oral. Cir. Bucal. 16 e306-11.
[4] Krishna Rao S V, Mejia G, Roberts-Thomson K and Logan R 2013 Epidemiology of oral cancer in Asia in the past decade--an update (2000-2012). Asian. Pac. J. Cancer Prev. 14 5567-77.
[5] Huang S H and O'Sullivan B 2013 Oral cancer: Current role of radiotherapy and chemotherapy. Med. Oral. Patol. Oral. Cir. Bucal. 18 e233-40.
[6] Kovács A F, Döbert N and Engels K 2012 The effect of intraarterial high-dose cisplatin on lymph nodes in oral and oropharyngeal cancer. Indian. J. Cancer. 49 230-5.
[7] Nwe N, Furuike T and Tamura H 2014 Isolation and characterization of chitin and chitosan from marine origin. Adv. Food. Nutr. Res. 72 1-15.
[8] Wimardhani Y S, Suniarti D F, Freisleben H J, Wanandi S I and Ikeda M A 2012 Cytotoxic effects of chitosan against oral cancer cell lines is molecular-weight-dependent and cell-type-specific. Int. J. Oral. Research. 3 e1.
[9] Wimardhani Y S, Suniarti D F, Freisleben H J, Wanandi S I, Siregar N C and Ikeda M A 2014 Chitosan exerts anticancer activity through induction of apoptosis and cell cycle arrest in oral cancer cells. J. Oral. Sci. 56 119-26.
[10] Ma P L, Lavertu M, Winnik F M and Buschmann M D 2009 New insights into chitosan-DNA interactions using isothermal titration microcalorimetry. Biomacromolecules. 10 1490-9.
[11] Amir L R, Suniarti D F, Utami S and Abbas B 2014 Chitosan as a potential osteogenic factor compared with dexamethasone in cultured macaque dental pulp stromal cells. Cell. Tissue. Res. 358 407-15.
[12] Weltrowski A, da Silva Almeida M L, Peschel D, Zhang K, Fischer S and Groth T 2012 Mitogenic activity of sulfated chitosan and cellulose derivatives is related to protection of FGF-2 from proteolytic cleavage. Macromol. Biosci. 12 740-50.
[13] Booth L A, Tavallai S, Hamed H A, Cruickshanks N and Dent P 2014 The role of cell signalling in the crosstalk between autophagy and apoptosis. Cell. Signal. 26 549-55.
[14] Dagenais M, Douglas T and Saleh M 2014 Role of programmed necrosis and cell death in intestinal inflammation. Curr. Opin. Gastroenterol. 30 566-75.
[15] Elmore S 2007 Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35 495-516.
[16] Luo H, Li J and Chen X 2010 Antitumor effect of N-succinyl-chitosan nanoparticles on K562 cells. Biomed. Pharmacother. 64 521-6.