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A comparison of the cytotoxic activity of cisplatin versus l-amino acid oxidase on squamous cell carcinoma cell line

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ABSTRACT

Objectives: This study used flowcytometry to compare the cytotoxic effect of cisplatin and L-amino acid oxidases (LAAOs) on the HEP-2 cell line 24- and 48-hours following treatment. *Methods:* This prospective laboratory study was conducted at the Applied Research Unit, Vaccine and Serum Research Institute, Cairo, Egypt and King Abdulaziz University- faculty of dentistry, Jeddah, Saudi Arabia in July 2017.The HEP-2 cell line was cultivated and cisplatin and LAAO were added to the culture medium. Flowcytometry was used to assess the cells 24 and 48 hours following treatment. *Results:* We found that LAAOs had a greater cytotoxic effect than cisplatin against the HEP-2 cell line, compared to cisplatin. The apoptosis rate was directly proportional to the duration of exposure. *Conclusions:* Our analyses show that LAAO has a stronger cytotoxic effect on HEP-2 cells than cisplatin, highlighting the potential of LAAO in the treatment of squamous cell carcinoma. Although our findings still need to be validated in other models, our findings strongly suggest that LAAO may cause substantial tissue damage.

Keywords: Squamous cell carcinoma cell line, HEP-2, Cisplatin, L-aminoacid oxidase LAAO, Flowcytometry

1. INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) typically arise in the oral cavity and pharynx and comprise over 90% of neoplasms of the head and neck region (Hedberg and Grandis, 2015). These cancers are associated with a high mortality rate, and in spite of advances achieved in the medical and surgical treatment of cancers, no improvements in mortality have been documented during the past four decades. Mortality rates of as high as 60% have been reported in patients receiving standard treatment comprising radiation therapy, surgery, and/or chemotherapy (Licitra et al., 2004).

Clinicians are still facing considerable challenges in treating cancer, which remains a major cause of mortality worldwide (Jemal et al., 2011). Challenges in achieving good outcomes in cancer patients are partly due to the aggressiveness and metastatic potential of various cancer types as well as the host's defense mechanism. Currently available treatment options—surgery, chemotherapy and radiotherapy - are inefficient and have their downside in that they also affect normal cells (Gomes et al., 2010; Tolstonog and Simon, 2017).

For several decades, investigators have searched for compounds from natural products (plants and animals) that have an anti-neoplastic effect, and until date, scientists are still investigating the use of purified chemicals in treating cancer (Mathan et al., 2018). Scientists studied the underlying molecular mechanisms and signaling pathways in oncogenesis to develop novel targeted anticancer therapies (Kozakiewicz and Grzybowska-Szatkowska, 2018; Puram and Rocco, 2015; Seebacher et al., 2019). These agents have several properties: some inhibit the vascular endothelial growth factor, epidermal growth factor, receptors, and matrix metalloproteinases. Conversely, other agents promote apoptosis (Seebacher et al., 2019; Wang et al., 2015) .

Apoptosis refers to the process of controlled and regulated cell death that is necessary in the development and maintenance of complex organisms. Several morphological and biochemical hallmarks characterize this process. These include phosphatidyl serine exposure on the surface of the plasma membrane, condensation of the nucleus, and chromatin fragmentation into oligonucleosomes (Julian and Olson, 2015). Apoptosis is a physiologic process that occurs during development and aging, and it is necessary to maintain cell populations in tissues. Cell death also occurs in response to an immune attack or during disease- or toxin-related cell destruction (Hongmei, 2012). Cisplatin was the identified compound in platinum-containing anti-neoplastic agents, which also comprises carboplatin and oxaliplatin (Apps et al., 2015). It cross links DNA and interrupts cell division in a variety of ways. A series of processes are triggered in an attempt to repair the damaged DNA, and when these fail, apoptosis is activated (Hongmei, 2012). Although the use of cisplatin was a major achievement in HNSCC treatment of HNSCC, tumor response and patient outcomes were still unsatisfactory. This prompted investigators to explore other chemotherapeutic agents, which can be combined with cisplatin to improve response and survival (Pendleton and Grandis, 2013).

Snake venom comprises a complex mixture of pharmacologically active substances, such as metalloproteases (Slagboom et al., 2017), phospholipases A_2 (Bordon et al., 2012), serine proteases (Roldán-Padrón et al., 2019), and other important enzymes. The substances in snake venom have neurotoxic, hemorrhagic and coagulant properties and can cause a range of adverse reactions, some of which are potentially life-threatening (Kandiwa et al., 2018). L-amino acid oxidases (LAAOs) are found in some venomous snake families (Mackessy et al., 2018). In some snake species, these proteins comprise close to one-third of the total venom proteins (Izidoro et al., 2014). Several investigators have identified the *in vivo* activity of LAAOs. Wei et al. (2009) demonstrated that the injection of 5µg LAAO induced paw edema in laboratory animals. Other investigators showed LAAOs induced hemorrhages (Kandiwa et al., 2018) and generalized effects, such as kidney toxicity (Vikrant et al., 2017). Further, some investigators showed that LAAO-treated cells initially underwent autophagy, while apoptosis and necrosis subsequently occurred (Costal-Oliveira et al., 2019). *Invitro* studies have demonstrated that LAAOs possessed antibacterial (Ciscotto et al., 2009; Sun et al., 2010), leishmanicidal (Rodrigues et al., 2009), and trypanocidal properties (Izidoro et al., 2014). Furthermore, these proteins were shown to be toxic to cancer cell lines (de Melo Alves Paiva et al., 2011) and could induce and / or inhibit platelet aggregation. Other investigators reported a correlation between these effects and the synthesis of hydrogen peroxide (Sun et al., 2010).

Previously, it was shown that LAAOs had an antineoplastic effect against squamous cell carcinoma (Wael et al., 2017). In the study, LAAO was reported to be the main cause of apoptosis in the HEP-2 cell line, with the apoptosis percentage being time-dependent. The objective of this research is to compare the anti-neoplastic activities of LAAO and cisplatin on squamous cell carcinoma.

2. SUBJECTS AND METHODS

Material

Squamous cell carcinoma cell line (HEP-2) was provided by the Cell Culture Department, Vaccine and Serum Research Institute, Egypt. The American Type Culture Collection shipped HEP-2 cells in a frozen vial (reference number, HTB-96). Cisplatin was used as a cytotoxic agent and was bought from Sigma Aldrich, USA. L-amino acid oxidase was used as a cytotoxic agent and was also bought from Sigma Aldrich, USA.

Methods

Tissue culture was conducted at the Applied Research Unit, Vaccine and Serum Research Institute, Cairo, Egypt and King Abdulaziz University- faculty of dentistry, Jeddah, Saudi Arabia in July 2017.

1. HEP-2 cell line propagation

Human laryngeal squamous cell carcinoma (HEp2) cells, produced by the Egyptian Organization for Biological Products & Vaccines (VACSERA®), were obtained for this experiment. The cells were grown under sterile conditions in a 50cm^3 flask containing Dulbecco's modified Eagle's medium. This medium included 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in 95% air and 5% carbon dioxide at 37°C.

2. Use of MTT reagent to determine cytotoxicity

This was done to determine the concentration at which LAAO and cisplatin allowed 50% of the cells to be viable.

Assay protocol

This consisted of culturing HEP-2 cells in 100 ml of the culture medium in a flat-bottomed 96 well plate. In every well, 10 ml of MTT reagent was added before incubating the plate for three hours. After adding Detergent reagent to each well to make the formazan dye soluble, the absorbance of all the samples was measured in a microplate reader set at a wavelength of 550–600 nm based on filter availability.

Figure 1 shows that concentrations of cisplatin that allowed 50% viability of HEP-2 cells were750 µg/ml at 24 hours and 375 µg/ml at 48 hours. Figure 2 show that the concentrations of LAAO that allowed 50% viability of HEP-2 cells were25 µg/ml at 24 hours and 12.5 µg/ml at 48 hours.

3. HEP-2 cell line treatment

Propagated HEP-2 cells were divided into six groups:

- Group I and II: HEP-2 cells untreated and cultured for 24 and 48 hours (control group).
- Group III and IV: HEP-2 cells treated with 375 µg/ml of cisplatin for 24 and 48 hours, respectively.
- Group V and VI: HEP-2 cells treated with 12.5 µg/ml of LAAO for 24 and 48 hours, respectively.

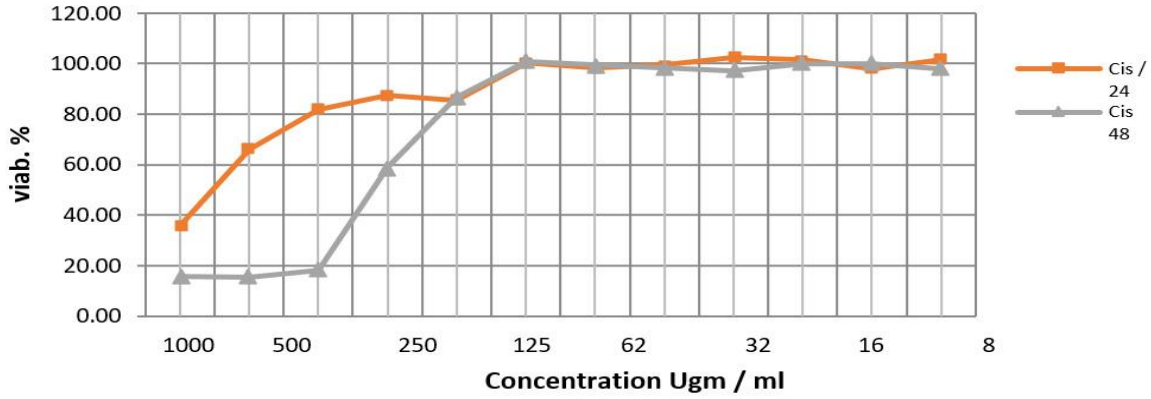

Figure 1 Concentrations of cisplatin that allowed 50% viability of HEP-2 cells 48 hours after treatment

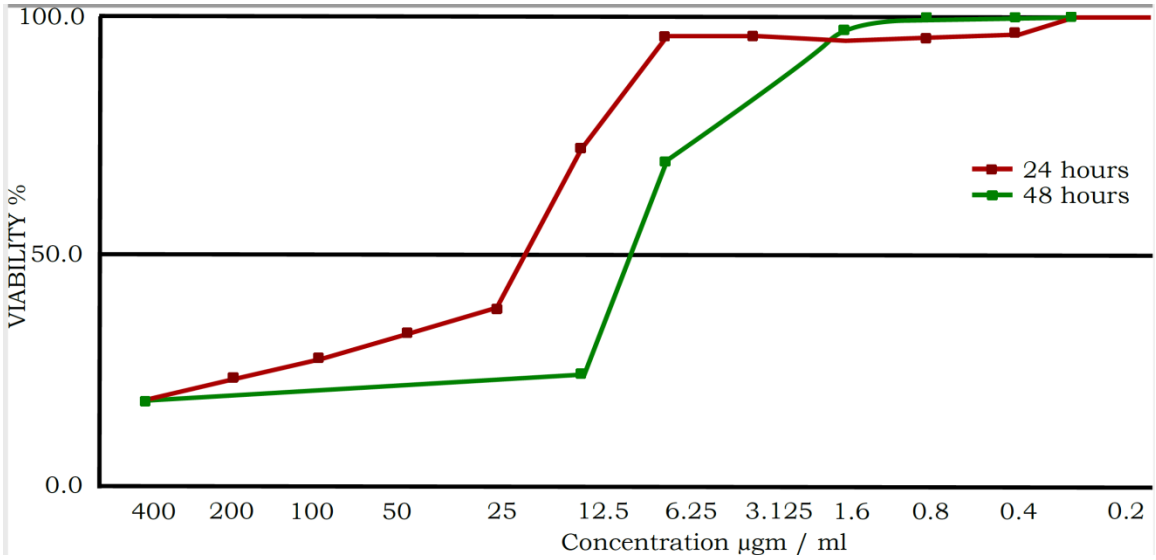

Figure 2 Concentrations of L-amino acid oxidase that allowed 50% viability of HEP-2 cells 48 hours after treatment

4. Flowcytometry technique

Cellular apoptosis was detected using the Annexin V-FITC kit. Annexin V binds to phosphatidyl serine in early apoptosis, while propidium iodide is an indicator of late apoptosis and necrosis.

Phosphate-buffered saline was used to wash the cell samples, which were centrifuged for 5 minutes at 50xg at 4ºC. The supernatant was decanted, and the pelleted cells were suspended in a binding buffer. Next, 1µL of Annexin V-FITC solution and 5µL of dissolved propidium iodide were gently mixed with the cell suspension. The tubes were placed on ice, in a poorly lit area, for 15 minutes. Binding buffer (400µL) was poured into the tube, which was stirred gently to mix its contents. Flowcytometry was used to analyze the cell preparation.

Ethical Consideration

Ethical approval were obtained from King Abdulaziz University- faculty of dentistry, Jeddah, Saudi Arabia

3. RESULTS

I. Control group

Approximately 99.98% of HEP-2 cells were viable after 24 hours without treatment and 87.1% of the cells were viable after 48 hours. Apoptosis was observed in only0.02% of the cells after 24 hours compared to 12.9% of the cells that showed early apoptotic changes after 48 hours. Late apoptosis or necrosis was detected after 24 and 48 hours (Figure 3).

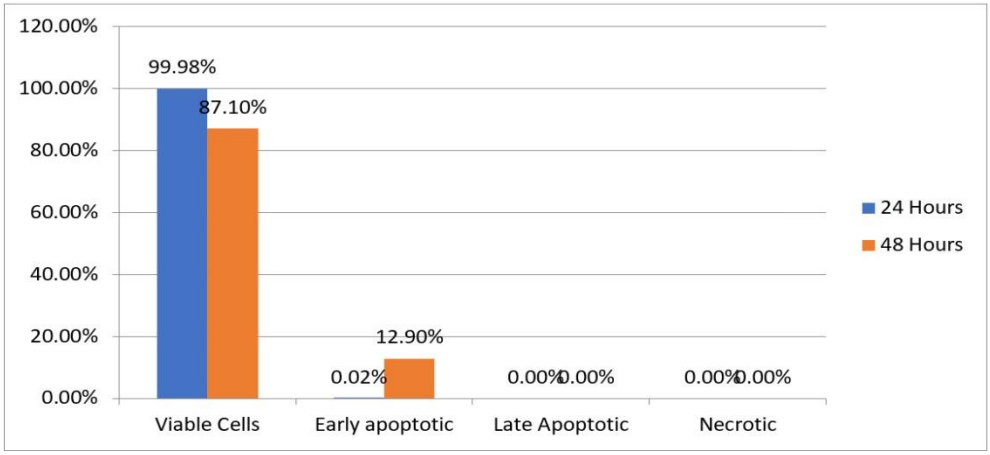

Figure 3 Proportion of apoptotic cells in the control group

II. Treatment of HEP-2 cells after 24 hours

The percentage of viable cells after using LAAO and cisplatin was 46.4% and 90.7%, respectively. Early apoptotic changes were detected in 53.7% of the cells treated with LAAO and 8.6% of those treated with cisplatin. The cell lines did not show substantial late apoptosis or necrosis after treatment with both agents (Figure 4).

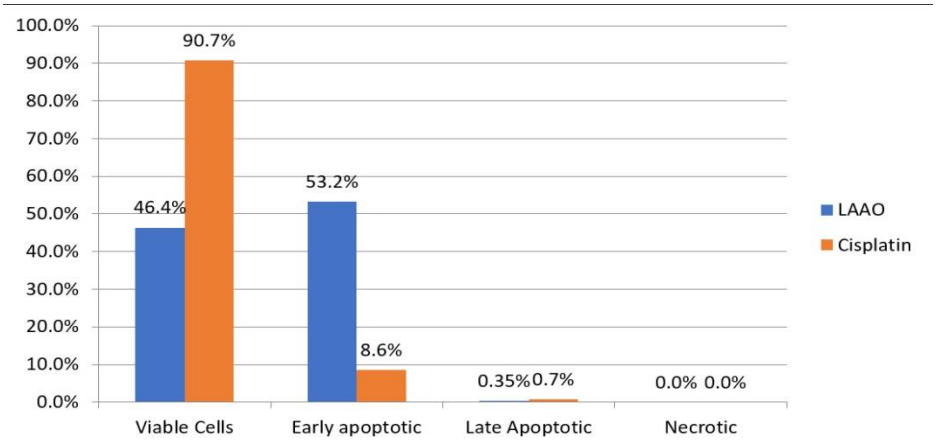

Figure 4 Proportion of apoptotic cells after 24 hours of treatment

III. Treatment of HEP-2 cells after 48 hours

Only 16.7% of the cells were viable after using LAAO, whereas up to 78.2% of the cells were viable after treatment with cisplatin. Approximately 83.4% and 18.6% of the cells, respectively, treated with LAAO and cisplatin showed early apoptosis. Late apoptotic changes were detected in 3.3% of the cells after using cisplatin; only 0.8% of the cells treated with LAAO showed apoptosis. Necrosis was not detected with both agents 48 hours after treatment (Figure 5).

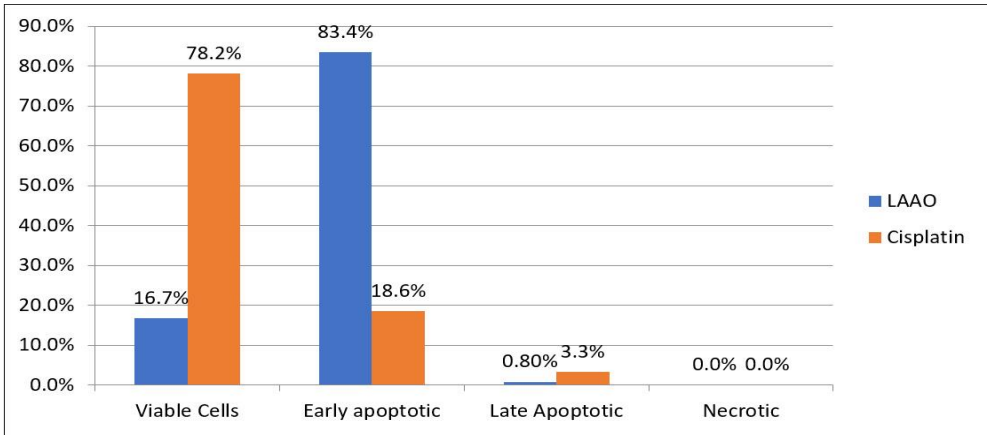

Figure 5 Proportion of apoptotic cells after 48 hours of treatment

4. DISCUSSION

Head and neck squamous cell carcinomas are relatively common, accounting for 600,000 new cancer cases diagnosed annually in the world. It is ranked as the sixth most common cancer worldwide and is associated with a high mortality rate (Bray et al., 2017). Several risk factors have been identified and include smoking, alcohol consumption, and human papilloma virus infection (Dhull et al., 2018).

Although several treatment options are available for patients with head and neck cancer, clinicians are still faced with the difficulty of selecting the appropriate treatment option for each patient. Additionally, while scientists have a better knowledge and understanding of the epidemiology and pathogenesis of HNSCC, sustainable improvements in the survival rates of patients are poor (Cadoni et al., 2017). Researchers are still faced with the challenge of developing effective treatments for cancer, which is still one of the leading causes of death worldwide (Bray et al., 2017).

As researchers gained new insights on the apoptotic process, new parameters were defined for the detection and measurement of apoptosis. Phosphatidyl seriene expression on cell surfaces is amongst these newly defined parameters. Cell membrane integrity is preserved during the early phase of apoptosis, but this is subsequently lost as membrane phospholipids (Zhang et al., 2018). Phosphatidyl seriene becomes exposed at the cell surface and serves as a signal that helps macrophages recognize and remove apoptotic cells (Lee et al., 2011). Among the initial signs that characterize the apoptotic process is the loss of plasma membrane asymmetry. In cells undergoing apoptosis, phosphatidyl serine is translocated from the inner to the outer leaflet of the plasma membrane, leading to the exposure of the phospholipid to the extracellular environment (Nonaka et al., 2017). Annexin V is typically used to detect apoptosis early. Some investigators used Annexin V-FITC as a fluorescent probe and showed that phosphatidyl seriene exposure was a nearly event of apoptosis, which occurred before alterations in DNA and membrane leakage. Thus, annexin V binding is beneficial in the detection of cell death early on during the process (Demchenko, 2013).

We determined the proportion of HEP-2 cells that underwent cell death by apoptosis using the Annexin-V FITC kit. The proportion of HEP-2 cells that underwent apoptosis 24 hours after treatment was higher with LAAO than with cisplatin administration (53.7% versus 8.6% for cells treated with cisplatin). Similarly, the proportion of HEP-2 cells that underwent apoptosis after 48 hours was higher in cells treated with LAAO than those treated with cisplatin (83.4% versus 18.6% for cells treated with cisplatin). These findings may be explained by the prolonged exposure of HEP-2 cells to increased hydrogen peroxide concentrations. In previous reports, it was found that leukemia cell lines (Jurkat and K562) underwent apoptosis when treated with a low concentration of LAAO. Conversely, the cells underwent necrosis when exposed to higher concentrations of the enzyme (Ande et al., 2006; Samel et al., 2006). The role of hydrogen peroxide in LAAO cytotoxicity was highlighted in a recent report where researchers found that catalase and other hydrogen peroxide scavengers could disrupt activities that induced apoptosis (Costal-Oliveira et al., 2019). On the other hand, LAAO-induced apoptosis was differentiated from exogenous hydrogen peroxide-induced apoptosis, suggesting that LAAO-induced apoptosis was not only triggered by hydrogen peroxide formed from oxidation (Teixeira et al., 2016).

5. CONCLUSION

Our analyses show that LAAO has a stronger cytotoxic effect on HEP-2 cells than cisplatin, highlighting the potential of LAAO in the treatment of squamous cell carcinoma. Although our findings still need to be validated in other models, our findings strongly suggest that LAAO may cause substantial tissue damage. Further research should investigate the efficacy of LAAO in cisplatin-resistant squamous cell carcinoma.

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Conflicts of Interest: The authors declare no conflict of interest.

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