Apoptotic effect of crude scorpion venom on head and neck squamous cell carcinoma cell line versus normal human epithelial cell line: In-vitro study

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Abstract--- Aim: The aim of this study was to investigate and compare the apoptotic effect of Leiurus quinquestriatus (LQ) scorpion venom on oral squamous cell carcinoma (OSCC) and normal human epithelial cell lines, in an attempt to introduce a novel selective therapeutic modality for OSCC. Methodology: we explored the effect of LQ scorpion venom on OSCC cell line (HNO-97) compared to normal oral epithelial (OEC) cell line. The cell viability by MTT assay and the expression of PUMA by ELISA were analyzed. Cell cycle analysis was studied for both groups using flow cytometry. Finally, we compared the expression of ROS by ELISA. Results LQ scorpion venom showed selective cytotoxic and apoptotic effect on OSCC cells. In comparison to normal cells, significant dose dependent cell viability reduction, cell cycle arrest and increased expression of apoptotic marker PUMA as well as ROS were observed in cancer cell group in comparison to the control group. Conclusions: LQ crude venom exerts a strong cytotoxic effect against OSCC with minimal effect on normal epithelial cells, thus, providing a selective, effective and less toxic possible chemotherapeutic agent for such a malignancy.
Keywords---oral squamous cell carcinoma, scorpion, leiurus quinquestriatus, cell lines.

Introduction

Throughout the ages, nature has catered to the basic needs of humans, not the least of which is the provision of medicines for the treatment of a wide spectrum of diseases. Currently, more than 50% of the drugs used worldwide are derived from natural products (Choene & Motadi, 2016). The scorpion, which is one of the oldest creatures known, has existed on earth for more than 400 million years. Among the different species of scorpions, the Buthid scorpion Leiurus quinquestriatus (LQ) is found throughout Egypt, Palestine, Syria, Jordan, and Kingdom of Saudi Arabia and considered one of the commonest and most dangerous species in these areas (Salama & Sharshar, 2013). In general, scorpion venom is considered to be a major health hazard. However, it has great potential if used in the correct way to fight various types of illness and is also being used by drug designing teams to synthesize an appropriate toxin-neutralizing vaccine (Al-Asmari et al., 2018).

In addition, in the past few decades, various studies have shed the light on the importance of scorpion venom as an anti-cancer agent in a variety of cancers. Salem et al. (2016) pointed out that not only the crude scorpion venom has exerted beneficial anti-cancer effect but also the purified peptides extracted from it too. Among the main effects exerted on cancer cells, induction of apoptosis and inhibition of cell progression as well as disruption of normal architecture of the cancer cells were reported (Diaz-Garcia et al., 2013). Despite the intensive focus on the effect of scorpion venom on different types of cancer, for the best of our knowledge, scarce studies in the available literature were conducted to examine the effect of scorpion venom on oral cancer. However, such cancer is considered as the 6th most common cancer globally and has a 5-year survival rate of around 50% according to US cancer statistics (Chen et al., 2018). In Egypt, the incidence rate of oral cancer ranges approximately from 1.4 to 2 per 100,000 persons (Ferlay et al., 2015). It's worth noting that above 90% to 95% of all cancers of the oral cavity is oral squamous cell carcinoma (OSCC) (Al-Jaber et al., 2016). Surgery, radiation, and chemotherapy in various combinations are utilized in the management of OSCC, depending on TNM stage and primary site. However, all of these treatments are associated with toxicity leading to late organs dysfunction (Marur & Forastiere, 2016).

At the molecular level, PUMA (p53-upregulated modulator of apoptosis) is the most potent apoptosis inducer, which is elevated in response to different stimuli through p53-dependent or independent transcription (Wang et al., 2007; Yan & Su, 2017). It also functions as a critical regulator of apoptosis in OSCC cells (Bin Hafeez et al., 2009). In the same context, reactive oxygen species (ROS) are relevant to both the pathogenesis and treatment of a variety of head and neck cancers including OSCC. Given the role that ROS play in carcinogenesis, they are appealing targets for intervention. Multiple reported molecules, many of natural origin, which target elements of redox pathways have been shown to alter growth
and/or progression of OSCC both in vitro and in vivo, including a significant number within the last two years (Kesarwala et al., 2016).

Accordingly, it seemed very interesting to explore the effect of LQ crude venom on oral cancer and normal cell lines, in an attempt to introduce a selective, novel efficient therapeutic modality for OSCC patients that may improve their quality of life. The viability and cell cycle analysis as well as the expression of apoptotic marker PUMA and ROS were examined using the state-of-the-art techniques: Methyl Thiazol Tetrazolium (MTT) assay, flow cytometry and the Enzyme-linked Immunosorbent Assay (ELISA), respectively.

Method

Cell lines

Two cell lines were purchased from Nawah Scientific Inc (Al-Mokattam, Cairo, Egypt) and supplied with compatible nutrient media. They were stored in liquid nitrogen containers at (-196°C). HNO-97: Human tongue carcinoma cell line and OEC: Normal oral epithelial cell line.

Scorpion venom

Highly purified (>97% purity) crude scorpion venom of Leiurus quinquestriatus scorpions was purchased from Egy Venom Company for Scorpion & Snakes Venoms Production for Research & Medical Uses, Cairo, Egypt. The venom was dissolved in sterile DMEM medium (20mg:1ml). Filtered venom was tested for sterility for 48 hours at 37°C using tryptic soy broth (Sigma Aldrich, USA).

The MTT viability assay and IC50 value calculation

All the reagents of the viability assay were purchased from Sigma Aldrich chemicals and were stored at 2-8 °C. Fixative: dimethyl sulphoxide (DMSO): isopropanol (1:1) (98%) Stain: 3-(4, 5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 5 mg/ml. Cytotoxicity screening was done on both normal & cancer cells to determine the cytotoxic effect of the crude venom. The untreated cells were used as a negative control. The cytotoxic dose that killed cells by 50 % (half maximal inhibitory concentration (IC50) was determined by using MASTER PLEX 2010 software. The cell viability percentage was calculated using the following formula: Cell viability % = (Mean optical density of treated cells)/(Mean optical density of negative control) ×100.

Annexin V& PI for flow cytometry

Fluorescein isothiocynate (FITC) conjugated form of Annexin (Annexin V-FITC Apoptosis Detection Kit, Catalog #: K101-25, Biovision, 980 Linda Vista Avenue, Mountain View, CA 94043 USA). Venom effect on cell proliferation was evaluated by measuring the distribution of cells in different phases of the cell cycle by flow cytometry. This assay is based on the identification of the DNA content of PI labelled nuclei according to the method of Vindeløv & Christensen with slight
modifications (Vindeløv & Christensen, 1990). All the procedures were carried out according to manufacturer’s instructions (www.biovision.com).

**PUMA ELISA kit**

Human PUMA ELISA Kit, Catalog No. MBS2500852 (MyBioSource, Inc. San Diego, CA 92195-3308, USA) is used in vitro for quantitative determination of PUMA in serum, plasma and other biological fluid according to manufacturer’s data sheet (www.mybiosource.com).

**ROS ELISA kit**

Human ROS ELISA kit Catalogue Number: AMS.E01R0021 (AMSBIO, 184 Park Drive, Milton Park, Abingdon OX14 4SE, UK. is a 1.5 hour solid-phase ELISA designed for the quantitative determination of Human ROS. According to manufacturer’s data sheet (www.amsbio.com), this ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti ROS antibody and an ROS HRP conjugate.

**Study design**

Cell lines were sub-cultured to obtain the study groups (4 groups). Both cell lines were subjected to the crude venom (t/HNO-97 and t/OEC), the un-treated cells were used as control (c/HNO-97 and c/OEC). The dose of the venom was determined using the MTT viability assay. Then, cell cycle phases and apoptosis analysis in the different study groups were assessed using flow cytometry. Furthermore, ROS and apoptosis indicator, PUMA, were measured by ELISA.

**Statistical methods**

The statistical package for the social sciences (SPSS) version 26 was used to code and enter the data (IBM Corp., Armonk, NY, USA). The mean and standard deviation were used to summarize the data. In the normally distributed quantitative variables, analysis of variance (ANOVA) with multiple comparisons post hoc test was used (Chan, 2003). P value ≤ 0.05, was considered significant.

**Results**

**Toxicity and cell viability using MTT assay**

The cell viability percentage in both groups (HNO-97 and OEC) showed gradual decrease with increasing concentrations of the venom. The lowest value of cell viability was found at the highest concentration of the venom (2000 μ/ml), while the highest value of cell viability was found at the lowest concentration of the venom (1μ /ml). It’s worth noting that the IC50 value of the venom in OEC group (140) was almost double that of HNO-97 group (74.5) (Figure 1).
**Expression of apoptotic marker PUMA using ELISA**

The highest levels of expression of PUMA were detected in t/HNO-97 group, followed by the c/HNO-97 group. On the other hand, the c/OEC group showed the lowest value of PUMA expression. ANOVA test revealed a high statistically significant difference among all the study groups. Multiple pairwise comparisons between the t/HNO-97 versus all other groups showed a statistical significance (Figure 2).

**Expression of ROS using ELISA**

Both treated cell lines yielded a higher values of ROS than their controls. However, t/HNO-97 group showed a marked higher ROS expression than the t/OEC group. ANOVA analysis among the four experimental groups showed highly significant statistical difference in levels of ROS expression with a P value < 0.001 (Figure 3). All the post hoc pairwise comparisons showed high statistically significant differences on comparing each two groups together except for c/HNO-97 versus c/OEC, which showed non-significant difference.

**Cell cycle analysis and determination of apoptosis**

Our investigations revealed that the venom caused a reduction in percentage of t/HNO-97 cells in the G0/G1 phase. Also, it inhibited transition of cells towards S-phase and allowed the increase in the proportion of cells in G2/M phase. An obvious accumulation of cells in G2/M and subG1 phase can be observed from 3.16% & 1.63% in c/HNO-97 group to 37.39% & 19.75% in the t/HNO-97 group, respectively. Alterations in the distribution patterns of cell cycle phases were also observed in t/OEC group; being increased in G2-M (12.14%) and pre-G1 phase (3.69%) and decreased G0-G1 (57.39%) & S phase (30.47%) in comparison to untreated group. All obtained data are represented a column chart (Figure 4, 6).

The highest % of total apoptosis was seen in t/HNO-97 group (10.82 late apoptotic subpopulation, 6.31 early apoptotic cell subpopulations and 2.62 necrotic cells). On the contrary, the untreated group showed very low mean % of total apoptosis (0.85 early apoptotic subpopulation, 0.42 late apoptotic cell subpopulations and 0.37 necrotic cells). However, the lowest % of total apoptosis was seen in c/OEC (Figure 5,6,7). Statistical Analysis of total apoptosis showed a high significant difference among all the study groups. The same trend in the percentage of early and late apoptosis as well as necrosis was observed among all groups. Multiple pairwise comparisons of total apoptosis between the t/HNO-97 group versus all other groups showed highly significant difference. Furthermore, the t/OEC compared to other groups revealed a non-significant value, except versus t/HNO-97 group. The same was obtained when the average values of each incident were compared.
Figure 1. A linear graph showing viability % of cells at different concentrations of the venom

Figure 2. Column chart showing expression of PUMA among the experimental groups

Figure 3. Column chart showing the expression of ROS among the experimental groups
Figure 4. Column chart showing DNA content at various phases of cell cycle among the experimental groups.

Figure 5. Column chart showing the mean % of apoptotic cells in experimental groups.

Figure 6. DNA histograms showed that apoptotic cells were almost undetectable in both untreated groups (b & d) and treated normal cell group (c). The % of apoptotic cells was highest in the t/HNO-97 (a) followed by t/OEC group (c).
Figure 7: Dot plot representation of flow cytometric analysis showed that most of cells in all study groups located in the first quadrant as they were viable. An increase in percentage of cells in early apoptotic phase (located in second quadrant) and in late apoptotic phase (located in third quadrant) was observed in t/HNO-97 group (a). Necrotic cells (located in fourth quadrant) were also detected in all study groups, with the highest percentage in t/HNO-97 group (a)

Discussion

The use of scorpion venoms for inhibition of cancer growth and induction of apoptotic cell death have shown in a growing number in-vitro and in-vivo studies (Moradi et al., 2018). Considering these promising results and aiming to introduce an available anticancer agent derived from the surrounding nature, scorpion venom was used as a subject in our study. Moreover, particularly LQ scorpion was chosen because it is the most common scorpion species in Egypt and belongs to the highly toxic and medically important family “Buthidae” (Sarhan et al, 2020). For the best of our knowledge, this is the first report showing the effect of LQ venom on OSCC compared to normal oral epithelial cells. Scarce studies examining the effect of other types of scorpion venom on OSCC were found in the available literature, with only two studies (both used BmK\-2 peptide) worked on dental pulp stem cells (Arpornsuwan et al., 2014; Satitmanwiwat et al., 2016) and gingival epithelium (Satitmanwiwat et al., 2016) as normal controls.

The induction of cytotoxic effect is a key mechanism of inhibition of the uncontrolled cell growth which is a hallmark of cancer development. Our results revealed that LQ venom showed a dose dependent reduction in cell viability in both types of cell line. In accordance with our results, many researches proved
the dose dependent cytotoxic effect of LQ venom on cell lines derived from different cancers such as breast and prostate cancers (Omran, 2003), breast and colorectal cancer cell lines (Al-Asmari et al., 2018), prostate cancer (Elfiky et al., 2019), and breast, hepatocellular, colon, cervix and alveolar adenocarcinoma cell lines (Salama & El-Naggar, 2021).

Furthermore, in order to establish an appropriate dose of venom, IC50 values of LQ venom on cancer and normal cell lines were determined. The IC50 of venom in the HNO-97 group was estimated as 74.5 μg/ml denoting a potential cytotoxic effect of LQ venom on OSCC cells. Interestingly, LQ venom showed a highly selective cytotoxic effect against cancer cells rather than normal cells, as the normal cell group showed almost the double IC50 value. This selective cytotoxic action is in agreement with Satitmanwiwat et al. (2016) which attributed this selectivity to the presence of specific molecules on the cell surface because one of the major differences between cancer and non-cancer cell external surfaces is the exposure of the negatively charged molecules.

At the molecular level, LQ scorpion venom induced a significant over expression of PUMA in HNO-97 in comparison to normal and untreated groups, which suggests that apoptotic activity might be linked to the p53-dependent mitochondrial pathway. In the same context, Diaz-Garcia et al. (2017) reported that PUMA and other members of Bcl-2 family showed similar results in treated breast cancer cell line with R. junceus scorpion venom. The over-expression of these apoptotic-related genes resulting in increased mitochondrial membrane permeability. This in turn, causes the release of cytochrome c into the cytoplasm followed by caspase-9 activation which directly activates the catalytic enzyme caspase-3 or caspase-7 and triggers the DNA fragmentation (Strasser et al., 2011).

The phenomenon of cell cycle arrest has been used as a therapeutic tool to inhibit tumor progression in in vitro studies (Badr et al., 2012). In this context, the reduced percentage of cells after application, which was observed in G0/G1 and S phases in treated cancer group, may be due to changes in the cancer cells and their DNA content as preparatory steps for programmed cell death. Chien et al. (2010) observed similar results after treatment of OSCC cell line with cobra venom. Moreover, several studies showed that naturally derived compounds from different plants produced the same reduction of oral cancer cells in this phase (Kwan et al., 2016; Yu et al., 2016; Wang et al., 2017).

On the contrary, a highly significant increase in the G2/M cell percentage was observed in the cancer group, which suggests that cell cycle arrest occurred at this phase. This finding reflects the beneficial effects of the proposed treatment in the current work. This is in agreement with Salama & El-Naggar (2021) who observed a similar change in the G2/M phase in colon cancer cell line after LQ venom treatment. Also, Al-Asmari et al. (2018) observed a similar enrichment of G2/M and cell cycle arrest in venom-treated colorectal cancer and breast cancer cell lines. However, colorectal cancer cell line showed a different pattern of cell cycle progression when treated with a different scorpion venom and all phases were enriched. They attributed this variability to the different genetic makeup of the cells in addition to the efficacy of venoms against a specific cancer cell type.
The cytotoxic effect of different scorpion venoms may be attributed to immune response modulation (Yang et al., 2000), ionic channel blockage (Wang & Ji, 2005), membrane integrity disruption (Gupta et al., 2007), polysaccharides fragmentation (Feng et al., 2008) and mitochondrial damage and DNA destruction (Gupta et al., 2010). However, in the current work, we proposed that the cytotoxic effect of LQ venom depends on the presence of the extensively reported CTX peptide. Previous studies have shown that CTX binds to many cancer cells including melanoma, lung carcinoma, neuroblastoma, medulloblastoma, and glioma (Dardevet et al., 2015). They also reported that unlike other venom-derived compounds, CTX has a remarkable ability to bind to cancer cells without affecting healthy ones. This selective action is presumably due to the overexpression of CTX targets in cancer cells including certain ion channels and membrane proteins.

Finally, it was suggested that treatment of cancer cells with scorpion venom will create a stressful environment. As a result of this stressful event, free radicals generation and ROS formation occurs (Al-Asmari et al., 2018). To elucidate this, we determined the extent of ROS generation under the influence of LQ venom on the studied cell lines. A dramatic increase in the ROS generation was detected in venom-treated cancer cells when compared to normal epithelium and untreated cells. Our results are in strong agreement with Al-Asmari et al. (2018). They noted that ROS is a well-known player in the induction of DNA damage and also known to disrupt normal protein function, which leads to cell death. Moreover, Brenneisen & Reichert (2018) in their study on nanoparticles revealed that treatment of cancer cells with ROS generating agent might exceed a certain ROS threshold, thus resulting in detrimental oxidative stress which can’t be compensated by the defense systems of these cancer cells. In contrast, normal cells are often able to counteract increased ROS levels by their healthy endogenous antioxidant systems.

Taken together, our findings of decreased cell viability, increased ROS and PUMA expression, and cell cycle arrest at G2/M phase in OSCC cell line strongly suggest that LQ scorpion venom has an anticancer potentials against OSCC. The venom kills cells by different mechanisms but the relationship between these mechanisms is still unclear. Moreover, it exhibits a minimal toxicity against normal epithelial cells, making it a promising selective chemotherapeutic agent. This is likely to be confirmed by further investigation. This study provides supportive data for future investigations that may lead to their use in cancer therapy.

**Conclusion**

LQ scorpion venom had cytotoxic effect on OSCC cell line in a dose dependent manner and caused cell cycle arrest at G2/M phase and induced apoptosis during Pre-G1 in OSCC cell line. Moreover, LQ scorpion venom induced apoptosis probably via elevated levels of PUMA and ROS. Thus, LQ scorpion venom had a selective effects on OSCC cell line rather than normal epithelial cell line making it a potential selective chemotherapeutic agent.
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Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

References


