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THERAPEUTIC POTENTIAL OF EGYPTIAN COBRA SNAKE VENOM ON HUMAN OSTEOSARCOMA CELL LINE

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ABSTRACT

Background: Osteosarcoma is a primary bone malignancy with a poor prognosis, often requiring novel therapeutic approaches. Snake venom has shown potential anticancer properties, targeting various cancer types.

Objective: This study evaluated the therapeutic potential of Egyptian cobra (Naja haja) venom on the human osteosarcoma MG-63 cell line, focusing on its cytotoxic, apoptotic, and cell cycle arrest effect.

Material and Methods: MG-63 cells were treated with varying concentrations of Egyptian cobra venom, and cell viability was assessed using an MTT assay. Morphological changes were observed through histological analysis and nuclear morphometry. Cell cycle distribution and apoptosis induction were analyzed using flow cytometry and Annexin V/PI staining, respectively.

Results: Egyptian cobra venom exhibited dose-dependent cytotoxicity against MG-63 cells, with pre-IC_{50} of 62.5 μg/ml, IC_{50} of 125 μg/ml, and post-IC_{50} of 250 μg/ml. Treated cells showed morphological features of apoptosis and necrosis. Nuclear morphometric analysis revealed significant changes in nuclear area factors across treatment groups (***P*** value < 0.001). Flow cytometry demonstrated G0/G1 phase arrest, with the percentage of cells in G0/G1 increasing from 54.13% in pre-IC_{50}, and 61.22% in the IC_{50} to 69.04% in the post-IC_{50} group. Annexin V/PI staining showed a dose-dependent increase in apoptosis, from 18.29% in the pre-IC_{50} and 23.61% in the IC_{50} to 35.11% in the post-IC_{50} group.

Conclusion: Egyptian cobra venom showed significant anticancer potential against osteosarcoma cells by inducing apoptosis and cell cycle arrest. These findings suggest its potential as a novel therapeutic agent in osteosarcoma treatment.

KEYWORDS: Osteosarcoma; Egyptian cobra venom; Naja haja; Cytotoxicity; Apoptosis; Cell cycle arrest; MG-63 cells

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INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor, primarily affecting children and adolescents. The prognosis of osteosarcoma patients, even with advanced surgery and chemotherapy, is still unsatisfactory, especially for metastatic or recurrent cases [1].

Interest in natural bioactive compounds, including snake venoms has been escalating over the last few decades searching for more potent and less toxic novel therapies than currently available [2].

The potent biochemical effect of snake venoms has been well documented and in recent years there has been a detailed analysis of their anti-cancer properties. Several studies have demonstrated that venom components such as phospholipase A2 (PLA2), neurotoxins, and cytotoxins can target cancer cells, induce apoptosis, and disrupt tumor progression [3].

Interestingly, some snake venom components demonstrate mechanisms of action similar to current chemotherapy agents. For example, Lebein, a snake venom disintegrin, exhibits an anti-angiogenic effect by inhibiting vascular endothelial growth factors (VEGF) [4].

Additionally, the Caspian cobra venom has been shown to induce mitochondrial and caspase-3-dependent apoptosis in cancer cell lines with minimal effect on normal cells [5].

Egyptian cobra (Naja haja) venom, a complex mixture of proteins and enzymes, has shown promise in treating various cancers, including hepatocellular carcinoma and lung cancer [6].

However, its effect on osteosarcoma cells has not been extensively studied. The current study aimed to explore the cytotoxic and apoptotic activity of Egyptian cobra venom using the osteosarcoma MG-63 cell line. We aim to offer a new perspective on these applications by revealing their induction of cell death and cell cycle arrest in osteosarcoma.

MATERIAL AND METHODS

Ethical Approval

The research protocol with protocol number (852 \ 2023) was approved by the Research Ethics Committee of the Faculty of Dentistry, Minia University at meeting number (101)

MATERIAL

Cell Line and Culture Protocol

The human osteosarcoma MG-63 cell line was purchased from NAWAH Scientific (Egypt). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidified incubator. The cells were treated with different concentrations of Egyptian cobra snake (Naja haja) venom to determine pre-IC_{50}, IC_{50}, and post-IC_{50} doses.

Venom Preparation

Venom was obtained from the Egyptian Organization of Biological Products and Vaccines (EGYTOX, Egypt) and stored at 4°C. Venom concentrations ranging from 0.05 μg/ml to 1000 μg/ml were prepared for experimental treatments.

METHODS

Cell Viability Assay

Methyl Thiokol Tetrazolium (MTT) assay was performed. MTT assay was performed to assess cell viability after treatment with varying concentrations of venom (0.05-1000 μg/ml) for 24 hours.

Histological Analysis

Cells were stained with hematoxylin and eosin (H&E) to evaluate morphological changes.

Photomicrographs were taken using an Olympus BX60 microscope with a magnification of 100X oil.

Nuclear Morphometric Assay

Nuclear morphometric analysis was conducted using image analysis software (ImageJ, NIH, USA). Parameters such as nuclear circularity and nuclear area factor (NAF) were calculated for the control, pre-IC_{50}, IC_{50}, and post-IC_{50} groups.

Flow Cytometry for Cell Cycle Analysis

Cell cycle distribution was analyzed using flow cytometry. Cells were stained with propidium iodide, and the distribution across G0/G1, S, and G2/M phases was recorded. The proportion of cells in each phase was compared between the control and venom-treated groups.

Annexin-V/Propidium Iodide Technique

Apoptosis and necrosis were evaluated using annexin-V and propidium iodide staining. The cells were analyzed by flow cytometry to determine the percentage of cells in early apoptosis, late apoptosis, and necrosis.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Differences between groups were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. A ***P***-value < 0.05 was considered statistically significant.

RESULTS

Cell Viability Assay

The cytotoxic effect of Egyptian cobra (Naja haja) venom on MG-63 osteosarcoma cells was evaluated using the MTT assay. Treatment with venom concentrations ranging from 0.05 μg/ml to 1000 μg/ml for 24 hours resulted in a dose-dependent decrease in cell viability (Table 1).

TABLE (1) Cell Viability (%) of MG-63 Osteosarcoma Cells Treated with Egyptian Cobra Snake Venom for 24 Hours

Ser	Sample	Concentration (μg/ml)	Viability %
1	**Pre-IC_{50} treated G.**	62.5 μg/ml	80%
2	**IC_{50} treated G.**	125 μg/ml	50%
3	**Post IC_{50} treated G.**	250 μg/ml	28%

Histological Analysis

Control MG-63 cells exhibited characteristics typical of malignancy, including cellular and nuclear pleomorphism and hyperchromatic nuclei (Figure 1A). In contrast, venom-treated cells displayed morphological features indicative of apoptosis and necrosis, with the effect becoming more pronounced at higher concentrations.

Pre-IC_{50} and IC_{50} treated groups showed signs of apoptosis, including cellular and nuclear shrinkage, irregular cell membranes, and formation of apoptotic bodies. Some cells also exhibited necrotic features such as ruptured cell membranes (Figure 1B and 1C).

The post-IC_{50} treated group demonstrated the most extensive cellular damage, with a higher proportion of cells showing advanced apoptotic and necrotic morphology, including nuclear fragmentation and the presence of necrotic debris (Figure 1D).

Nuclear Morphometric Assay

Nuclear morphometric parameters were analyzed across the treatment groups. The Nuclear Area Factor (NAF) showed a non-linear trend across the groups as illustrated in Table (2).

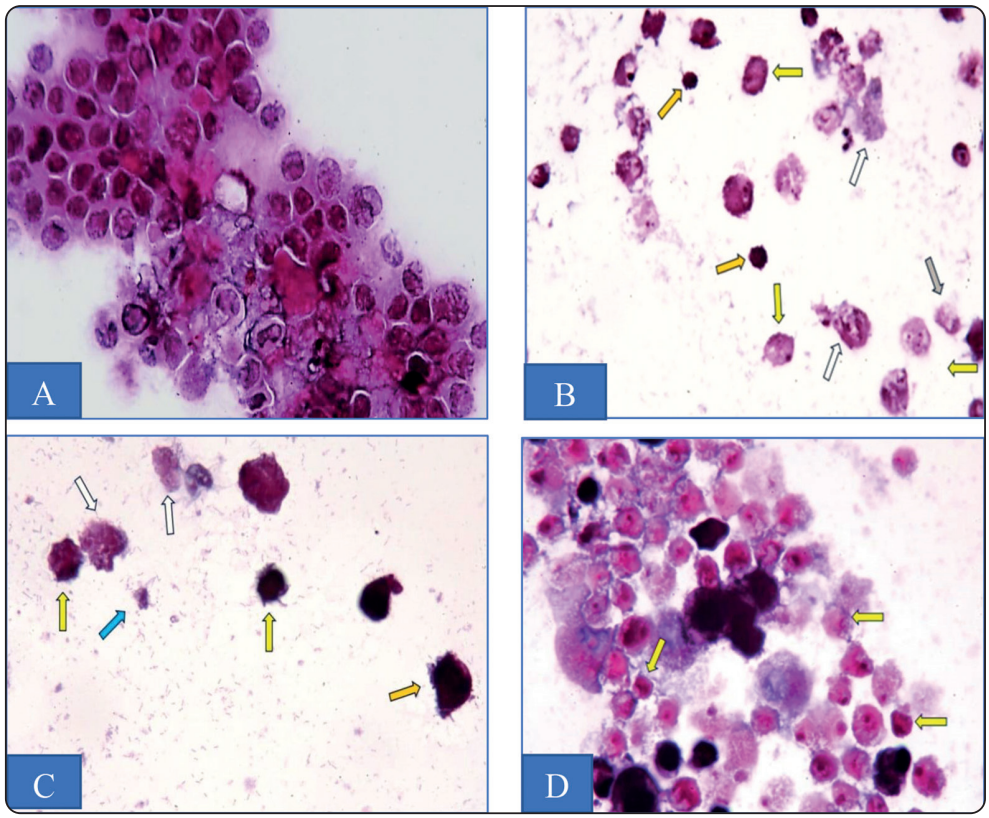

Fig. (1) Histological evaluation of MG-63 osteosarcoma cells showing: (A) Control cells, (B) Cells treated with pre-IC50 venom concentration, (C) Cells treated with IC50 concentration, and (D) Post-IC50 treated cells, (H&E) 100X oil magnification.

TABLE (2) Nuclear Morphometric Parameters of MG-63 Osteosarcoma Cells Treated with Egyptian Cobra Snake Venom

Ser	Group	Nuclear Circularity	Object Area (μm^2)	NAF
1	**Control Group**	0.3869	29182	11290.5
2	**Pre-IC_{50} treated G.**	0.3656	24902	9104.17
3	**IC_{50} treated G.**	0.3486	21069.49	7344.8242
4	**Post IC_{50} treated G.**	0.3049	15686	4782.66

Cell Cycle Analysis

Flow cytometric analysis revealed that Egyptian cobra venom caused cell cycle arrest at the G0/G1 phase in a dose-dependent manner. The percentage of cells in the G0/G1 phase increased from 48.07% in the control group to 69.04% in the post-IC_{50} group. Correspondingly, the percentage of cells in the S phase decreased from 38.12% in the control to 22.51% in the post- IC_{50} group, while the G2/M phase showed a slight decrease from 13.81% to 8.45%.as shown in Table (3).

Annexin-V/Propidium Iodide Technique

Annexin-V/Propidium Iodide technique demonstrated a dose-dependent increase in apoptosis and necrosis with increasing venom concentrations. Total apoptosis increased from 2.07% in the control to 35.11% in the post-IC_{50} group. Necrosis also increased from 1.55% in the control to 4.73% in the post-IC_{50} group as demonstrated in Table (4).

TABLE (3) Cell Cycle Distribution of Control and Egyptian Cobra Snake Venom-Treated MG63 Cells

Ser	Sample	%G0-G1	%S	%G2/M
1	**Control Group**	48.07	38.12	13.81
2	**Pre-IC_{50} treated G.**	54.13	32.66	13.21
3	**IC_{50} treated G.**	61.22	27.61	11.17
4	**Post IC_{50} treated G.**	69.04	22.51	8.45

TABLE (4) Annexin-V/Propidium Iodide technique Assay Showing Various Phases of Apoptosis and Necrosis

Group	Total Apoptosis (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)
Control Group	2.07	0.33	0.19	1.55
Pre-IC_{50} treated G.	18.29	10.05	4.92	3.19
IC_{50} treated G.	23.61	11.27	5.05	3.58
Post IC_{50} treated G.	35.11	15.11	19.11	4.73

DISCUSSION

Osteosarcoma (OS) is the most prevalent primary malignant bone tumor, ranking as the third most common malignancy in children and adolescents after lymphomas and brain tumors. It accounts for 6-7% of all OSs within the jaws and presents significant challenges in treatment and prognosis [7].

The complexity of OS necessitates a multidisciplinary approach to diagnosis and treatment, typically involving surgery, chemotherapy, and radiotherapy. However, the high metastatic potential of OS, with 10-15% of newly diagnosed patients showing metastasis, underscores the urgent need for novel therapeutic strategies [8].

In recent years, animal venoms have emerged as a promising source of bioactive molecules with potential anticancer properties. Snake venom, in particular, has garnered attention for its diverse biological effects, including antitumor activities [9].

The Egyptian cobra (Naja haja) venom, a complex mixture of proteins, peptides, and enzymes, has shown promise in various therapeutic applications [10].

Previous studies have demonstrated its potential in inducing apoptosis in cancer cell lines with minimal effects on normal cells, suggesting its potential as a candidate for cancer treatment [11-13].

The current study applied the multifaceted approach to investigate the effect produced by snake venom derived from Egyptian cobra on the behavior of osteosarcoma cell line MG-63.

The above results thus provided evidence for significant cytotoxic activities, changes in the cell morphology, and nuclear parameters inducing G0/G1 cell cycle arrest that followed apoptosis and even necrosis. This proves that the venom strongly inhibits the growth of osteosarcoma cells through anti-cancer properties.

Cell viability assays resulted in reduced cell viability of MG-63 osteosarcoma cells treated with Egyptian cobra venom in a dose-dependent manner. Thus, an IC_{50} value of 125 μg/mL was calculated

and caused about a %50 reduction in viability. Such data support the view that snake venoms can act cytotoxic against tumoral cell lines.

For instance, ***Omran et al. (2003)*** measured IC_{50}s for T47D and MDA-MB-468 breast cancer cells to be 18 μg/ml and 63 μg/ml, respectively, whereas IC_{50} of prostate cancer cells fell within the range of 38 to 61 μg/ml. Venom, at 100 μg/ml, induced significant cell death in T47D cells (98%) and MDA-MB-468 cells (75%) [14]

Kurkute et al. (2023) has demonstrated potent cytotoxic effect of Naja naja, with IC_{50} values 5-10 μg/ml for A549 lung cancer cells [15].

The main cytotoxicity of Naja haje venom results from components such as phospholipase A2 which disrupts cell membrane integrity and mocarhagin, a metalloproteinase that interferes with platelet adhesion and immune responses [16].

The cytotoxins further disrupt the integrity of the membranes of cancer cells to lyse lysosomes, while α-neurotoxins impose neuromuscular inhibition through the blockade of acetylcholine receptors [11].

This dose-dependent destruction in this present study may be expected from the concentration of these molecules.

Histologically, there were significant morphologic changes in the MG-63 cells after the administration of Egyptian cobra venom. The control cells exhibited typical malignant features represented by cellular and nuclear pleomorphism with hyperchromatic nuclei.

The venom-treated cells, however, showed features of apoptosis and necrosis represented by shrinkage of nuclei, irregular membranes, and apoptotic bodies.

These results align with ***Lafnoune et al. (2021)*** study who reported Moroccan cobra venom's ability to reduce tumor spheroid size and cause histological changes, including cell shrinkage and nuclear condensation in hepatocellular carcinoma [17].

Neweigy et al. (2022) observed similar effect with Naja haje venom, noting membrane disruption, nuclear fragmentation, and increased apoptosis [11].

The transition from apoptosis at lower concentrations to necrosis at higher doses in our study suggests a dose-dependent dual mechanism, consistent with findings from other venom research [18].

Nuclear morphometric analysis revealed notable changes in nuclear parameters among the treatment groups. The post IC_{50} group exhibited the lowest nuclear circularity (0.3049) compared to the control (0.3869), indicating increased irregularity in nuclear shape often a sign of cellular stress and early apoptosis [19].

The Object Area, representing nuclear size, decreased initially in the pre-IC_{50} group (15,686 μm^2) from the control (21,069.49 μm^2), then increased in the IC_{50} group to 24,902 μm^2 and in the post-IC_{50} group to 29,182 μm^2. This trend agreed with a study indicating that during the early stages of apoptosis, there is a reduction in nuclear size by cobra venom, which then undergoes swelling during later stages, possible due to necrosis [20].

This pattern may reflect various stages of cellular response to venom: the initial decrease could indicate chromatin condensation in early apoptosis, while the later increase suggests nuclear swelling during late apoptosis or necrosis [21].

The Nuclear Area Factor (NAF) followed a similar trend, decreasing initially before rising but remaining below control levels. These changes in nuclear parameters support the observed morphological alterations and suggest that venom significantly impacts nuclear structure and organization.

Egyptian cobra venom caused G0/G1 phase cell cycle arrest in MG-63 cells, increasing G0/G1 from 48.07% in controls to 69.04% in the post-IC_{50} group, while reducing S and G2/M phases.

This dose-dependent effect suggests that higher venom concentrations enhance cytotoxicity through apoptosis and necrosis.

These findings align with studies showing that Bothrops jararaca and Bothrops erythromelas induce G0/G1 arrest and apoptosis via mitochondrial disruption and DNA damage [22].

Similarly, Bothrops venoms have shown significant cell cycle arrest in K562 human leukemic cells[21].

Additionally, snake venoms can be more effective than traditional chemotherapeutics, as seen with Montivipera xanthina and Leiurus quinquestriatus having lower IC_{50} values than Tamoxifen [23].

Cell cycle arrest may result from the inhibition of cyclin-dependent kinases (CDKs) and the activation of DNA damage checkpoints, leading to repair or apoptosis The increase in G0/G1 cells indicates these checkpoint responses to venom-induced stress [24,25].

The Propidium Iodide/Annexin-V staining assay indeed showed the induction of both apoptosis and necrosis in MG-63 cells after treatment with Egyptian cobra venom in a dose dependent manner [26].

Hence, the total apoptosis increased from 2.07% in the control group to 35.11% in the post-IC_{50} group, while necrosis increased from 1.55% to 4.73%, which corroborated the studies of snake venom-induced cell death. Studies on the venom of *Vipera lebetina turanica* in colon cancer cells indicated that exposure resulted in apoptosis using ROS and JNK pathways, where increased apoptotic death was related to higher levels of ROS and upregulation of death receptors such as DR4 and DR5 [26].

This mechanism is in concord with our findings and thus points to the possibility that the increased apoptosis, as observed in our study, is mediated through similar oxidative stress pathways initiated by the venom of Naja haje.

One study centered around the venom of the Egyptian cobra observed that it could cause both apoptosis and necrosis, which depended on factors like concentration and period of exposure [27].

This duality suggests that apoptosis is the major form of cell death in our findings, while necrosis also occurs but to a lesser extent. The predominance of apoptosis suggests that, indeed, snake venoms effectively induce cell death through ROS-mediated pathways, although higher concentrations or prolonged exposure to the venom could result in necrosis.

The action of the venom was able to cause both apoptosis and necrosis under such duality of its action mechanism; this may enhance its potency against tumors. A dose-response increase in apoptotic and necrotic cell death was in good agreement with cytotoxicity and morphological data and thus provided further evidence for the potent anticancer effect of the venom.

Finally, ANOVA testing revealed significant differences between groups ($\boldsymbol{P} < 0.001$). Also, Post-hoc analysis showed significant differences between the control and pre-IC_{50} groups and between the post-IC_{50} and pre-IC_{50} groups ($\boldsymbol{P} < 0.05$).

CONCLUSIONS

This study demonstrated that Egyptian cobra (Naja haja) snake venom exhibited a potent anticancer effect against the human osteosarcoma cell line MG-63.

The venom induced dose-dependent cytotoxicity, significant morphological changes, alterations in nuclear parameters, cell cycle arrest, and both apoptotic and necrotic cell death.

The multi-faceted effect of the venom suggested that it may be effective against cancer cells that have developed resistance to conventional therapies.

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