

RESEARCH ARTICLE

Potential Anticancer Activity of Caspian Cobra Venom Through Induction of Oxidative Stress in Glioblastoma Cell Line

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Abstract Despite advances in therapeutic strategies in the management of cancer, malignant glioma remains difficult to treat due to progressive resistance to conventional drugs. New studies made efforts to develop new anticancer agents from the screening of natural compounds. The biodiversity of venoms and their bioactive toxins makes them a special source for the development of novel therapeutic agents. The aim of the present study was to investigate the effect of *Naja naja oxiana* (NNO) crude venom on U87MG glioma cell line. Cellular viability and the generated amount of reactive oxygen species were determined by MTT and redox-sensitive dye DCFH-DA, respectively. A dose-dependent decline in viability of cells along with increase in generation of reactive oxygen species (ROS) occurred after the 24-h exposure to NNO venom. Incubation of RBC with NNO venom for 24 h indicated that hemolysis was not more than 6%. The results showed that NNO venom might act through the production of excess ROS, further disruption of mitochondrial function, and decrease in viability of

U87MG without changes in the integrity of RBC membrane. However, more investigations are needed to find out detailed mechanisms by which NNO venom inhibits the viability of U87MG.

Keywords Caspian cobra venom · *Naja naja oxiana* · U87MG glioma cell line · Cytotoxicity · Reactive oxygen species

Introduction

Central nervous system tumors are the second most common cause of mortality in adult and second most common type of cancer in children [1]. Glioblastomas are one of the most lethal types of brain cancer, and due to their special biological features, they are resistant to radiotherapy and chemotherapy and not thoroughly removed via surgical operation [2]. In addition, administration of conventional chemotherapeutics often leads to serious side effects [3]. In this sense, novel anticancer drugs developed from natural resources may increase the efficacy of conventional chemotherapeutic drugs [4]. Many researchers believe that pharmacologically active components of snake venom are useful biological resources for the treatment of cancer [5]. It has been shown that snake venoms can potentially reduce the progression of solid tumors and angiogenesis [6] via induction of apoptosis in various cancer cell lines [7].

Naja naja oxiana (NNO), the Caspian cobra, is a highly venomous species of cobra in the family Elapidae found in Central Asia, and its venom have several biological functions such as anticancer and induction of apoptosis [8, 9]. It has been shown that NNO venom initiates apoptosis process by excessive production of reactive oxygen species (ROS) in colorectal cancer cells [10]. Disruption in cellular

Significance statement The authors identified that the *Naja naja oxiana* venom inhibits the viability of glioma cells through induction of the reactive oxygen species without any hemolytic effects on isolated red blood cells. Taken together, the present findings opened up interesting questions to find out the main mechanisms to the anticancer activity of this venom.

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redox status and defense system due to excess ROS may cause oxidative damage in vital cellular macromolecules and subsequently induces apoptotic signaling [11, 12]. In the present study, the viability of U87MG glioma cell line and the level of ROS generation of NNO venom were evaluated to find out whether this venom has potential to reduce the number of viable glioma cells via induction of oxidative stress. On the other hand, a hemolysis assay was done on red blood cells (RBC) to rule out their adverse effects of venom on RBC.

Material and Methods

Materials

Human glioblastoma cells, U87MG (ATCC[®]HTB-14[™]), were obtained from National Cell Bank of Iran. The cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Biowest, South America Origin) in a humidified incubator maintained at 37 °C with 95% O₂ and 5% CO₂. All of the other components were purchased from Sigma-Aldrich (St .Louis MO, USA).

Snake venom collection

Venom was extracted via snakebite through the parafilm stretched over a glass tube in the Department of Venomous Animals and Antivenom Production. All guidelines for the care and use of snakes as laboratory animals were approved by the Ethics Committee of Razi Vaccine and Serum Research Institute (Karaj, Iran). The extracted venom was centrifuged for 10 min at 500 g, and after removing the supernatant, the sediment was frozen in − 80 °C for 4 h and then lyophilized [13].

Cell viability assay

U87MG cells were seeded in 96-well plates at a density of 2×10^4 cells/well and after 24 h incubation; NNO venom (0.5–100 µg/ml) was added and incubated for extra 24 h. To carry out cytotoxicity assay, 20 µL of MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} (5 mg/mL) was added to each well and plates were incubated at 37 °C for 4 h. Then, formazan crystals were dissolved by 100 µL of dimethyl sulfoxide, and the absorbance was read at 570 nm and 630 nm (reference wavelength) with a microplate reader (BioTek Instruments, USA). The viability as the percent of control was calculated by following formula:

$$\text{Viability}\% = \frac{\text{OD } 570 - 630 \text{ nm test}}{\text{OD } 570 - 630 \text{ nm control}} \times 100$$

IC₅₀ value for NNO venom was obtained through Probit analysis by SPSS 16.0 for Windows software (Chicago, USA) [14].

Measurement of intracellular ROS

The redox-sensitive dye 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) was used to measure the level of intracellular ROS in U87MG. Cells were seeded at 3.0×10^4 cells/well and incubated for 24 h. Then, 200 µl H2DCF-DA (10 mM) was added to each well and incubated for 30 min at 37 °C in a dark room. After washing of well, cells were exposed to NNO venom (5–100 µg/ml) for 2 h. The fluorescence intensity was measured in a microplate reader at 485 nm excitation and 528 nm emission [15] and finally appropriate images were taken using a fluorescence microscopy.

Hemolysis assay

To draw a standard curve for hemolysis percent, hypotonic water lysis (hypotonic shock) was performed by adding up to 100 µl ice-cold distilled water to 10–50 µl of RBC taken from healthy Wistar rat and mixing them with vigorous agitation for 15 min. Then, the optical density of lysed RBC at 490 nm was plotted against its hemolysis percent. To evaluate the hemolytic effect of NNO venom, 50 µl of RBC was added into micro-tube containing 50 µl medium alone or plus different concentration of NNO venom and incubated for 24 h at 37 °C. After centrifugation, the absorbance of supernatants was read at 490 nm and hemolytic present calculated according to the standard curve.

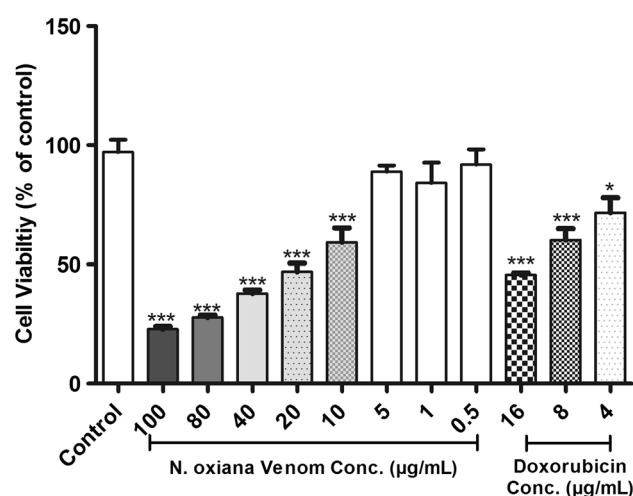


Fig. 1 Cell viability after treatment with different concentrations of *Naja naja oxiana* (NNO) venom (0.5–100 µg/ml) and Doxorubicin (4, 8, and 16 µg/ml) in U87MG cells. Values are mean $\pm$ SD of three experiments, each experiment performed in triplicate. *Differences between groups were significant at $p < 0.05$ ***Differences between groups were significant at $p < 0.001$

Statistical analysis

Data were analyzed by repeated measure ANOVA test followed by the Tukey posttest using GraphPad Prism 5.0 software (San Diego, USA), and a significance level of $p < 0.05$ was used for statistical testing.

Results and Discussion

Cytotoxic effect of NNO venom

As shown in Fig. 1, the NNO venom decreased the viability of U87MG in a dose-dependent manner through the

decrease in the mitochondrial reduction of tetrazolium salt. Viability of U87MG cells reduced to 90.8 ± 10.0 , 83.2 ± 10.0 , 89.9 ± 14.7 , 59.3 ± 14.6 , 46.4 ± 9.9 , 37.8 ± 3.8 , 27.8 ± 2.7 , 22.6 ± 3.2 of control after 24 h incubation of cells, with 0.5, 1, 5, 10, 20, 40, 80, and 100 $\mu\text{g/ml}$ of NNO, respectively, in a dose-dependent manner. The calculated IC_{50} for NNO venom and doxorubicin was $19.9 \pm 1.57 \mu\text{g/ml}$ and $16 \pm 1.2 \mu\text{g/ml}$, respectively. Ebrahim et al. [8] evaluated the effect of NNO venom on cell proliferation of some cancer cell lines and reported the involvement of this venom in mitochondrial-dependent apoptotic pathways and activation of caspase 3 with minimum effect on the normal cell line. They estimated the IC_{50} of this venom to be 26.59, 28.85, 21.17

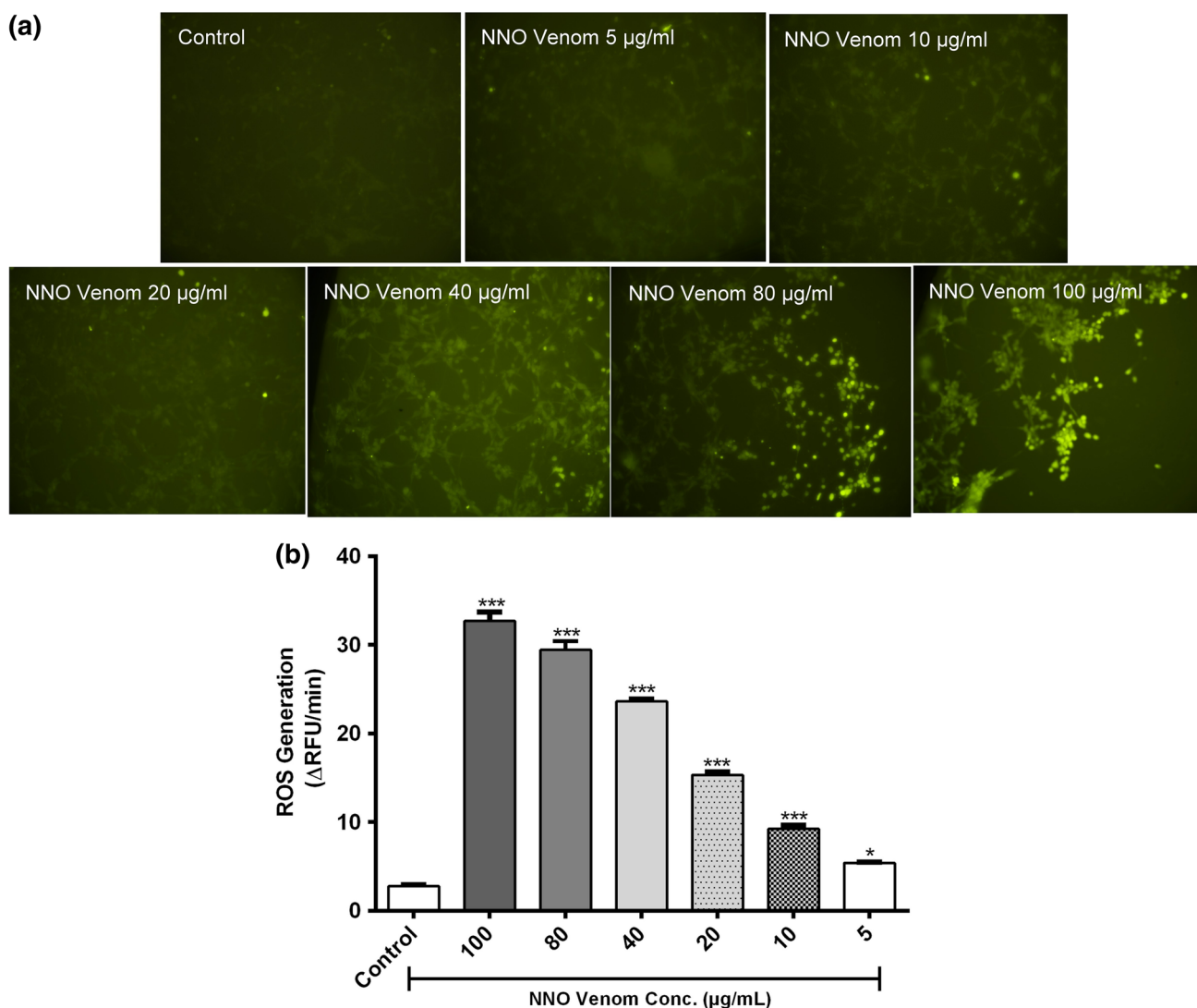


Fig. 2 **a** Fluorescence microscopy images of U87MG cells after 2 h exposure to different concentration of Naja naja oxiana (NNO) venom (5–100 $\mu\text{g/ml}$). **b** Generation of reactive oxygen species (ROS) as relative fluorescence units (RFU)/min after treatment with different concentrations of NNO venom (5–100 $\mu\text{g/ml}$) in U87MG cells.

Values are mean $\pm$ SD of three experiments, each experiment performed in triplicate. *Differences between groups were significant at $p < 0.05$. ***Differences between groups were significant at $p < 0.001$

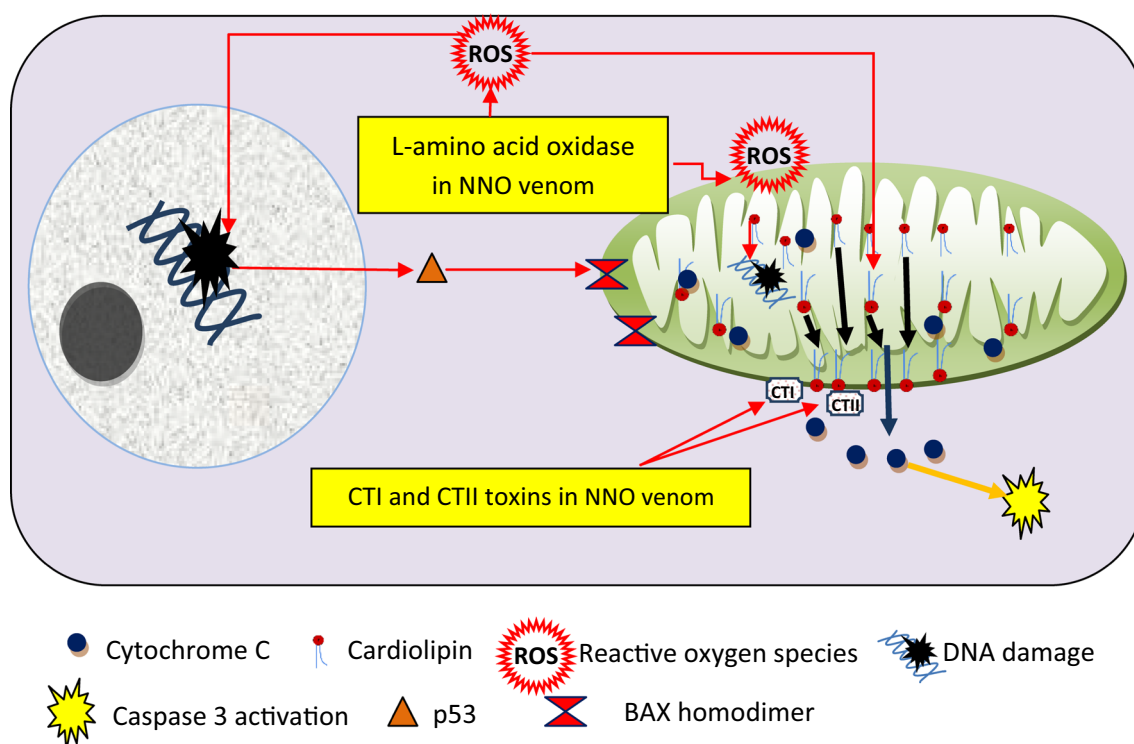


Fig. 3 Schematic diagram of mechanisms by which *Naja naja oxiana* (NNO) venom might poses its cytotoxic effects in cancer cells

and 47.1 $\mu\text{g/mL}$ for HepG2, MCF7, DU145, and MDCK cell lines, respectively. In another study, Fakhri et al. [10] showed ROS-mediated mitochondrial-dependent apoptosis in mitochondria obtained from colon cancer tissue after exposure to fractionated NNO toxin (IC_{50} , 20 $\mu\text{g/mL}$) and NNO venom (IC_{50} , 30 $\mu\text{g/mL}$). One mechanism contributed to disruption of mitochondrial membranes is binding of CTI and CTII, cytotoxins of NNO venom, to cardiolipin [16]. Cardiolipin is a phospholipid located in the inner membrane of mitochondria that can translocate to the outer membrane by oxidative stress and mitochondrial DNA damage caused by xenobiotics [17]. The translocated cardiolipins could consequently disrupt the membrane integrity and release mitochondrial contents such as cytochrome C [18]. Another mechanism proposed for mitochondrial toxicity and DNA damage potentials of NNO venom is the overproduction of ROS by NNO venom-treated mitochondria [10]. Mitochondria are a source of ROS production due to any change in mitochondrial oxidative phosphorylation chain and tricarboxylic acid cycle [19].

Effect of NNO venom on generation of intracellular ROS

In the present study, the authors observed that all of the concentration of NNO venom significantly increased ROS level in U87MG. ROS generation was increased significantly ($p < 0.001$) in response to all concentrations of

NNO in a dose-dependent manner (Fig. 2). This elevated expression of ROS could be attributed to the presence of L-amino acid oxidase (LAAO) in NNO venom which contributes to the generation of ROS mainly in the plasma membrane [20–22]. LAAO is a flavoprotein which produces H_2O_2 through de-amination of an L-amino acid and can induce subsequent cytotoxicity and apoptosis [23, 24]. Elevation of ROS-mediated oxidative injuries by inhibition of cellular antioxidant systems has a close relationship with tumor cell radio sensitivity [25] and drug sensitivity [26], which forms a target in killing of selective cancer cells. ROS is known to induce apoptosis under oxidative stress due to the presence of snake venoms in colorectal, breast, and the other cancer cell lines [8, 10, 27]. Despite mitochondria, other cellular sources including endoplasmic reticulum, cytosol, peroxisomes, and plasma membrane can generate ROS through the activity of enzymes, namely cytochrome P-450, oxidases, NO synthases, and lipoxygenases [28] that need to be evaluated about NNO venom. In order to illustrate the exact mechanisms involved in this toxin, evaluation of mitochondrial function and apoptosis pathways is suggested for future studies (Fig. 3)

Effect of NNO venom on hemolysis

Furthermore, the authors evaluated the hemolytic activity of NNO venom. Incubation of RBC with NNO venom for 24 h did not induce significant hemolysis at the

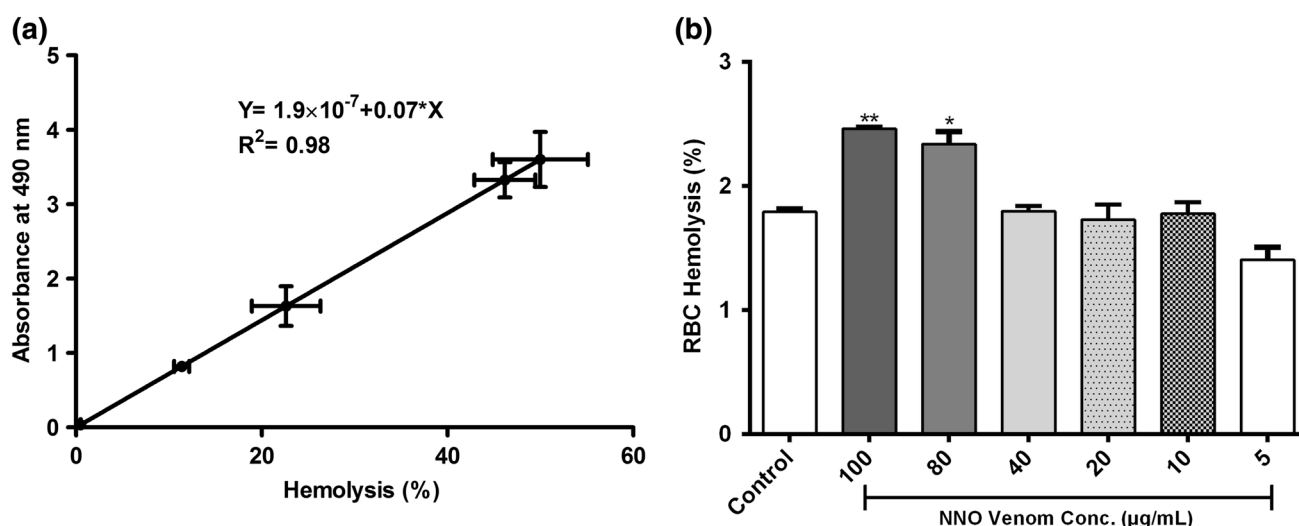


Fig. 4 **a** Standard curve for estimation of hemolysis percent of red blood cells (RBC) at 490 nm. **b** Percent of RBC Hemolysis after treatment with different concentrations of *Naja naja oxiana* (NNO)

concentration of 5–40 µg/ml. Higher concentration of NNO venom (80–100 µg/ml) led to the gradual increase in the percent of hemolysis up to $3.5 \pm 0.2\%$ (Fig. 4). An important issue in the treatment of cancer is the use of therapeutic strategies and drugs that have the least effects on normal cells. The first cells that are affected by chemotherapy are RBC, and evaluation of possible hemolytic effects of chemical and biological drugs is essential for the biocompatibility assessment of materials [29]. The hemolytic potential of NNO venom was less than 5% in comparison with control group. RBC hemolysis less than 5% is generally regarded as non-hemolytic and slightly hemolytic [29]. Therefore, NNO venom at concentration lower than 20 µg/ml has no possible toxicity on RBC.

Conclusion

Taking together, the authors conclude that the NNO venom generated ROS and decreased the ability of mitochondria in reduction of MTT in U87MG cell. ROS causes mitochondrial alterations triggering apoptosis signaling maybe through oxidative damage of cellular macromolecules. This venom induced cytotoxic effect in cancer cell without any changes in the integrity of RBC. Currently, various chemotherapeutic medications used for cancer treatment, which affect normal cells and are in association with many adverse side effects. It has been found that biological agents such as snake venom in the treatment of tumors can selectively inhibit cancer cells without harming the normal cell population. The main pathways by which the NNO venom induces oxidative stress and causes damage to mitochondria remain unknown. Further studies are needed to evaluate the

effects of NNO venom in the enhancement of sensitivity in cancer cells to chemotherapy and radiotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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