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Evaluation of the fractions from Caspian cobra venom on apoptosis of infected BHK-21 by Rabies Virus

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ABSTRACT

Rabies is zoonotic acute encephalitis that continuously kills thousands of people annually with almost 100 percent fatality. In the present study, apoptosis was investigated in BHK- 21 cell lines infected by rabies virus. Apoptotic cells are identified by fragmented and dense chromatin masses and evaluated by microscopic and statistical methods. In vitro apoptosis was time and dose-dependent in 24 to 72 hours of incubation in BHK-21cell lines; however, a marked reduction in the number of apoptotic cells was observed, especially at the lowest concentrations of F4 and F5 fractions, obtained by FPLC of crude *Naja naja oxiana* venom. The number of infected apoptotic cells in the presence of different concentrations of two fractions F4 (40, 30 and 20µg/ml) and F5 (40, 25 and 15µg/ml) of Caspian cobra venom are obtained by Hoechst staining. According to the obtained results, by decreasing the concentrations of F4 and F5 fractions, the apoptotic indices were decreased in each incubation time. The F5 fraction in comparison with F4 at the same incubation times (24, 48 and 72h) showed more effective on apoptosis of infected cells. The highest percentages (66.57% and 65.43%) of apoptotic cells which were recorded after 48 and 72 hours belong to 40µg/ml of F5 fraction respectively. Our observations have shown that the use of a specific fraction (F5) of cobra venom, in an efficient concentration and time can cause apoptosis of rabies-infected cells, so it can be hoped that this toxic fraction will be a candidate in treatment of Rabies virus proliferation.

1. Introduction

Rabies is an acute viral disease that causes encephalomyelitis in all warm-blooded mammals and humans. The disease is caused by the neurotropic virus (belonging to the family Rhabdovirus and genus Lyssavirus). These families are abundant in nature and cause more than 80 types of viral infectious diseases in vertebrates, invertebrates and plants. The virus is transmitted from the saliva of the rabid animal bites into the muscles, scratches on the surface of the skin or mucous membranes (eyes, mouth, etc.). The virus migrates through the peripheral nerves to the central nervous system, where it multiplies and then spreads through the autonomic nerves to other tissues, such as the salivary glands, kidneys, lungs, liver, skin, heart,

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and muscles. If the virus is transmitted to another animal, the integrity of the neural network must be maintained. It is possible that such a virus may use destructive strategies to prevent neuronal dysfunction, including preventing cell destruction by apoptosis because apoptosis would jeopardize the virus's infectious cycle (Baloul and Lafon, 2003). Apoptosis, is one of the primary processes in Cell death in which the cell remnants disappear as they are phagocytosed by surrounding cells (Lyone and Esther, 1999). The major physiological mechanism of cell removal is apoptosis, in which cells are silently removed under normal conditions when they reach the end of their life span or are damaged by certain internal or external stimuli (Houchins, 2000). Although the death signal may be regulated by gene expression, the process can be set in motion by diverse stimuli, such as genotoxic damage (chemotherapy and radiation), or cytokines deprivation (Baloul and Lafon, 2003).

The biological process of apoptosis involves maintaining cellular homeostasis and preventing the pathogenesis of many diseases. Activation of caspases, which are in the family of cysteine proteinases, is involved in the onset of apoptosis. Several studies have shown that apoptosis prevents the spread of viruses in the body and is an important host defense mechanism against infectious viruses (Fernandes et al., 2011). Snake venom is a complex compound with acidic properties that mainly contains proteins with enzymatic activity and molecular weight between 1030 and 1070 kDa. Proteins and peptides with toxic properties such as neurotoxins, cardiotoxins, cytotoxins, myotoxins, coagulants, anticoagulants and enzymes such as proteases, oxidases, phospholipases, etc. make up 90% to 95% of the dry weight of snake venom (Tu, 1977; Tu, 1991). In addition, snake venom contains mineral cations such as sodium, calcium, magnesium and small amounts of zinc, nickel, cobalt, iron and manganese (Tu, 1991; Chegeni et al., 2015; Saedi and Amin zadeh, 2018).

Several studies have suggested the use of some venom components as therapeutic and anticancer agents, as well as in the production of recombinant neurotoxins (Maristela et al., 1999; Wang et al., 2002). The presence of L-amino acid oxidases (LAAO) in snake venom has biological and medicinal effects, including causing platelet aggregation and induction of apoptosis, bleeding, and cytotoxicity due to snake venom. This protein has a great potential for developing antimicrobial, antiviral, antiparasitic, anti-tumor and anti-single cell agents and produces hydrogen peroxide as a catabolic product by catalyzing an oxidation reaction and reducing various groups of amino acids. This type of reactive oxygen species (ROS) appears to be responsible for the molecular effects of these enzymes (Vural et al., 2016).

In vitro and *in vivo* observations suggest that apoptosis may be a protective mechanism for neurons. Preservation and maintaining of the neural network, limitation of inflammation, and destruction of T cells that invade the central nervous system (CNS) in response to infection are key events for rabies virus (RV) neurosis and RV transmission to another animal (Consales and Bolzan, 2007). On the other hand, studies have shown that any factor that inhibits the normal growth and development of cells, such as exposure to toxic agents or cryopreservation, may lead to apoptosis (Chegeni et al., 2015), Clinically, the most common and important effect of Elapid envenomation in Africa is local tissue necrosis (Dehesa-Davila et al., 1995; Warrell et al., 1976).

The mechanism of local necrosis resulting from Elapid venom may be different from that of the viper. Elapid venoms, generally, are not rich in enzymes, particularly proteases, thought to be responsible for the tissue damage that follows viper bites. African cobra's bites produce necrotic effects that could be explained by the 60-70 amino acid polypeptides, known as cytotoxins (or in some cases, cardiotoxins), and myotoxins, such as phospholipase A2. There is no clear correlation between the composition of Elapid venoms and the incidence of local necrosis after their bites; however, the injected venom dose may be an important variable (Warrell, 1995). Egyptian cobra, *Naja haje*, is widely distributed in Africa and the Middle East. The LD50 of its venom is 0.12µg/g (Mohamed and Hanna, 1973).

Envenomation causes local pain and swelling, and may be associated with blistering at the bite site. Neurotoxic and systemic symptoms develop within few hours, and deaths have occurred within 6-16 hours after large

snakes' bites, despite the use of antivenom and mechanical ventilation (Warrell et al., 1976).

Several authors have reported that sublethal doses of *N. haje* venom induced potent histopathological, histochemical, and pathophysiological alterations in the heart, liver, kidney, and brain of rats (El-Fiky, 1999; Hassouna and Rahmy, 2001; Omran, 1997; Omran et al., 1997; Omran et al., 2000). These pathological changes included severe degrees of cellular damage concomitant with marked signs of both myolytic and coagulative necrosis (Omran et al., 1997; Rahmy and Hemmaid, 2000; Rahmy et al., 1995). Snake venom from *Vipera lebetina turanica* can cause apoptosis in many cancer cell lines, but there are no studies on the effect of snake venom apoptosis on human neuroblastoma cells (Laemmli, 1970). Despite the great advances in the understanding of morphological and biochemical alterations associated with chemical induced cell injury, the *in vitro* cytotoxic and anti-rabies effects of Caspian cobra venom at the cellular level has not been well characterized.

In this study, we analyzed the apoptosis induced by Caspian cobra venom fractions on infected BHK-21 cells by challenge Rabies virus strain-11 (CVS-11) at various time intervals, attempting to clarify the cytotoxic and viral effects of this lethal venom.

2. Materials and Methods

2.1. Chemical reagents

Hoechst solutions of 1.0µg/ml concentration (part of a family of blue fluorescent dyes used to stain DNA) from stock solution (1mg/ml). Doxorubicin solution of 3.0µg/ml as an apoptotic inducer (from stock 50mg/25ml vial; Doxorubicin, Pfizer).

2.2. Snake venom preparation

Freeze-dried Caspian snake (*Naja naja oxiana)* venom was obtained from the Laboratory of venom and therapeutic biomolecules, Pasteur Institute of Iran.

2.3. Cell culture and the virus preparation

BHK-21 cells and Rabies Virus (CVS-11) were obtained from National reference center for rabies, Pasteur Institute of Iran. The BHK-21 cells were obtained from National Cell Bank of Iran and cultured in DMEM supplemented with 5% heated inactive fetal bovine serum (FBS), and the antibiotics penicillin (10000µ/ml) and streptomycin (10000µg/ml). Cells were cultured in a culture T75 flask and incubated at 37 □ C/5% CO_2 in a fully humidified atmosphere.

2.4. Venom (Naja naja Oxiana) fractionation

Naja naja oxiana crude venom was fractionated by size exclusion chromatography (Fast protein liquid chromatography; FPLC) as described previously (Farzad et al., 2020). Concentration and molecular weights of peptide fractions were determined using Bicinchoninic acid method (Smart BCA protein assay kit, Intronbio-Korea) and Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) method respectively (Chakrabarty and Sarkar, 2016; Farzad et al., 2020).

2.5. Evaluation of non-toxic concentrations of peptide fractions

The evaluation of non-toxic concentrations of peptide fractions on BHK-21 cells has been showed that, F4 and F5 fractions in comparison with other FPLC fractions had less cytotoxic effects on those cells, based on MTT assay (Farzad et al., 2020; Takeuchi et al., 1991; Mosmann, 1983). The non-toxic concentrations of F4 and F5 were 30 and 25µg/ml respectively (Farzad et al., 2020).

2.6. Evaluation of Anti-rabies effects of peptide fractions

The non-toxic concentrations of F4 and F5 peptide fractions of *Naja naja oxiana* venom were evaluated for their inhibitory effects on rabies virus (CVS-11 strain) infection on a 96-well plates containing 5×10^4 BHK-21 cells per well were exposed to rabies virus (MOI: 0.1) for 1 h in order to allow virus attachment to the cells. MTT and focus forming assay (FFA) were used to determine the inhibitory effect of venom fractions on infected cells for 24, 48 and 72 h after infection (Farzad et al., 2020).

2.7. Apoptosis assays

The BHK-21 cells (5×10^4) were cultured on 96-well microplates using the same culture medium as previously mentioned, then were infected by CVS-11 rabies virus (MOI:0.1) and incubated for one hour to attach cells. The infected cells were treated with various concentrations of F4 and F5 fractions (F4:40, 30 and 20 µg/ml, F5: 40, 25 and 25 µg/ml respectively) and incubated at 37 °C for 24, 48 and 72 hours. Chromatin condensation and/or nucleus fragmentation were morphologically investigated by 1µg/ml Hoechst staining reagent (Sigma, St. Louis, MO, USA). The microplate was incubated at 37 °C for 15 minutes. After the incubation time and discarding the solutions, each well was washed by PBS 1× buffer. The morphology of the cells was observed under a fluorescent microscope at 40× magnification using standard Fluorescein filter set (UV: 330-400nm). Apoptotic cells were identified by their characteristic fragmented and condensed chromatin masses and by counting the number of apoptotic nuclei per number of total nuclei in the same microscopic field, apoptotic cells were quantified. In this study, Doxorubicin of 10µg/ml concentration was used as a cell apoptotic inducer. The wells with this inducer were used as positive control.

3. Results

3.1 Qualitative Morphological analysis of Apoptosis of infected BHK-21 cells treated with certain concentrations of F4 and F5 fractions

In vitro apoptosis of BHK-21 cells was analyzed both qualitatively and quantitatively. There are two techniques were used to detect the morphological changes in the apoptotic cells. The Fluorescein Apoptosis Detection System, uses a florescent dye such as Hoechst reagent. Hoechst staining of BHK-21 cells was done after incubation with different concentrations of *Naja naja oxiana* FPLC fractions (F4 and F5).

Apoptosis was time and dose-related in 24 to 72 hours of incubation in BHK-21cell lines; however, a marked reduction in this number was observed, especially at the lowest concentrations of F4 and F5 fractions after all incubation times.

Hoechst reagent is a nucleophilic dye which stains and identifies the nucleus of apoptotic and non-apoptotic cells. Apoptotic cells are characterized by a syncytium-like appearance and an increase in the density of their cellular contents. Nucleus pattern and non-apoptotic cells with normal nucleus morphological pattern could be detected by fluorescence microscopy. Untreated BHK-21 cells were homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogenous or slightly granulated cellular contents. However, after incubating the BHK-21 cells with peptide fractions (F4 and F5) of *naja naja oxiana* venom for 24 hours, the occurrence of various morphological abnormalities was observed. At the lowest concentration of F4 (20µg/ml) and F5 (15µg/ml), most of the cells lost their characteristic appearance and coalesced to such an extent, that individual cells could not be identified. Some cells showed obvious deterioration and deformation, while some cells coalesced and lost their common structure (Fig 1). At the highest venom concentration of F4 (40 µg/ml) and F5 (40 µg/ml), the disappearance of normal morphological characteristics was very obvious (Fig 1) with cells showing severe shrinkage and condensation of their cellular contents.

3.2 Quantitative analysis of Apoptosis of infected BHK-21 cells treated with certain concentrations of F4 and F5 fractions

Quantitative analysis of apoptotic indices was performed by counting the number of infected apoptotic cells after Hoechst staining. The number of infected apoptotic cells in the presence of different concentrations of two fractions F4 (40, 30 and 20µg/ml) and F5 (40, 25 and 15µg/ml) of *Naja naja oxiana* venom are illustrated in Figure 2., by considering the positive control (C+; cell and Doxorubicin as apoptotic inducer).

The apoptotic indices of BHK-21 infected cells incubated with 40, 30 and 20µg/ml of F4 fraction and 40, 25 and 15µg/ml of F5 fraction (Figure 3), were decreased from 1.18% to 0.39% and from 61.08% to 5.08% respectively, after 24 hours of incubation.

The apoptotic indices of BHK-21 infected cells incubated with 40, 30 and 20µg/ml of F4 fraction and 40, 25 and 15µg/ml of F5 fraction (Figure 4), were decreased from 0.71% to 0.43% and from 66.57% to 6.14% respectively, after 48 hours of incubation.

The apoptotic indices of BHK-21 infected cells incubated with 40, 30 and 20µg/ml of F4 fraction and 40, 25 and 15µg/ml of F5 fraction (Figure 5), were decreased from 10.71% to 3.29% and from 65.43% to 16.3% respectively, after 72 hours of incubation.

According to the results obtained during 24, 48 and 72 hours of incubation, by decreasing the concentrations of F4 and F5 fractions, the apoptotic indices were decreased in each incubation time. The F5 fraction in comparison with F4 at the same incubation times (24, 48 and 72 hours) showed more effective on apoptosis of infected cells. The highest percentages (66.57% and 65.43%) of apoptotic cells which were recorded after 48 and 72 hours of incubation belong to 40µg/ml of F5 fraction respectively.

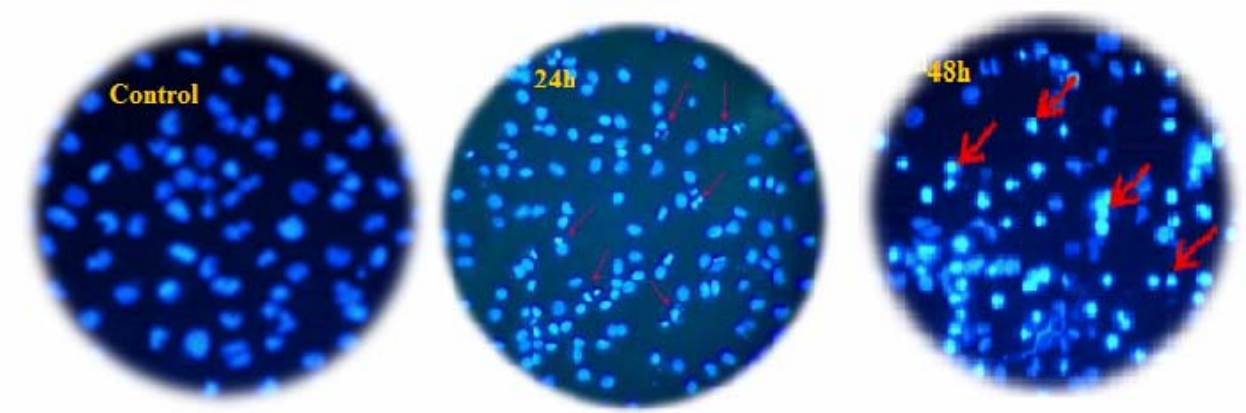

Figure 1. Morphological changes and the onset of nuclear fragmentation in infected BHK-21 cells treated with *Naja naja oxiana* venom after 24h and 48h incubation by fluorescence microscopy (magnification of 20X). Red arrows indicate infected cells with apoptosis; Apoptotic cells with specific morphological characteristics were seen as fragmented nuclei and dense chromatin.

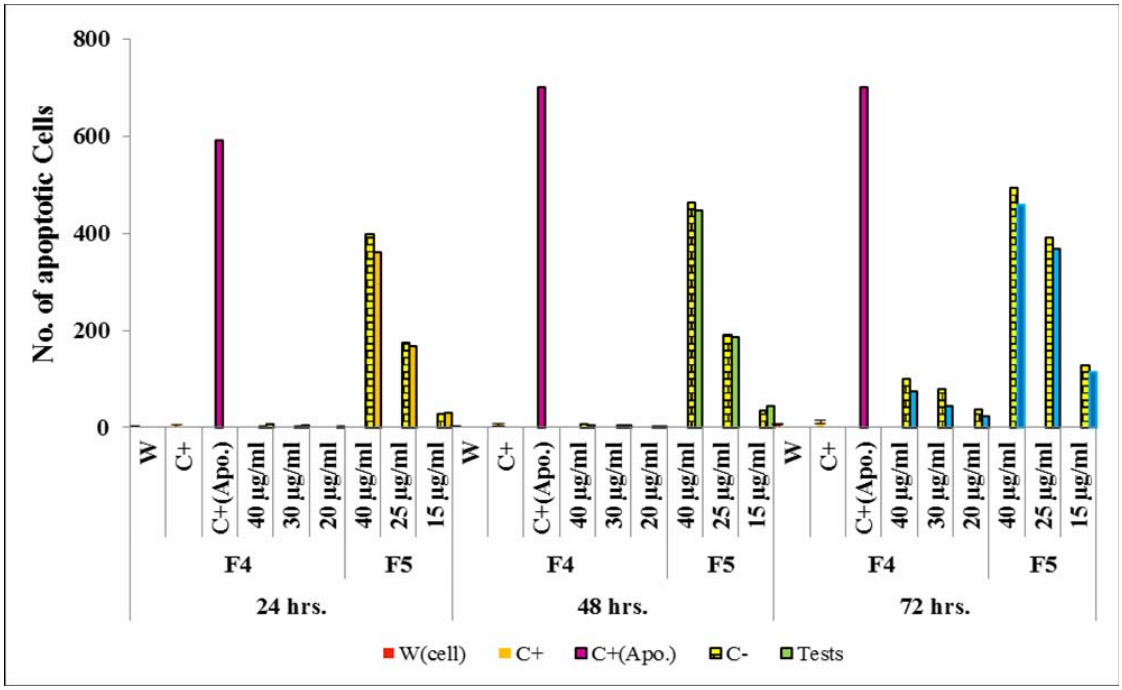

Figure 2. Simultaneous comparison of the effects of venom fractions (F4, F5) on apoptosis of Infected BHK-21 cell lines in three times of 24, 48 and 72 hours. W: cells without treatment, C+: cell and virus, C+ (APO): cell and apoptotic inducer (Doxorubicin), C-: Cell and snake venom fraction, Tests: Cell and virus and snake venom fraction

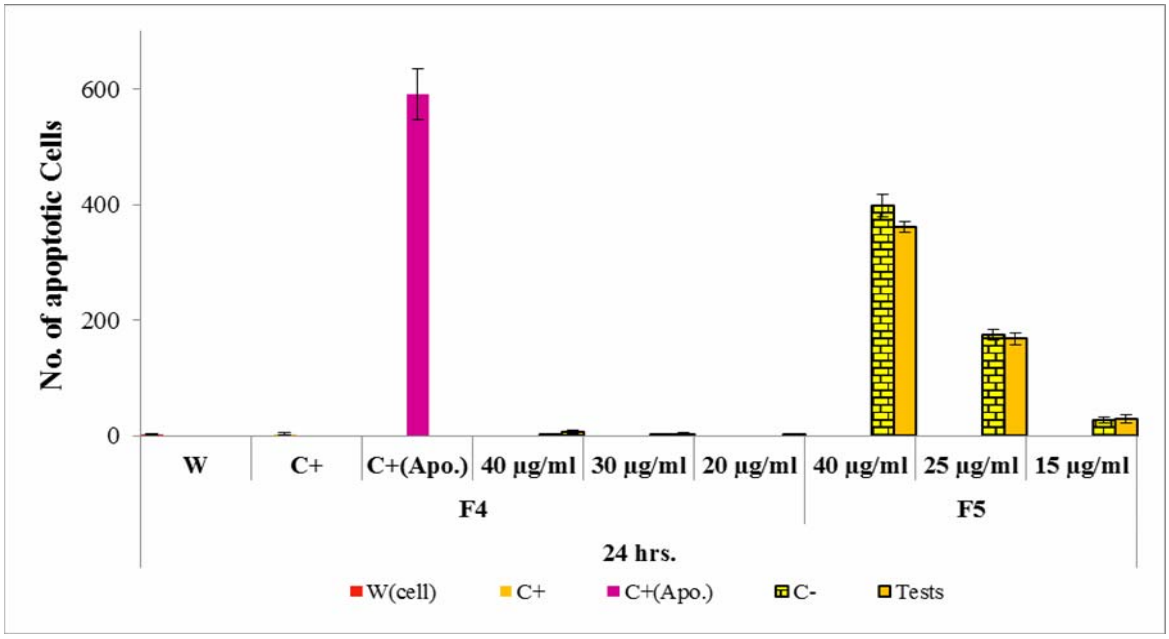

Figure 3. Evaluation of the effects of venom fractions (F4, F5) on apoptosis of Infected BHK-21 cells in 24h. W: cells without treatment, C^+: cell and virus, C^+ (APO): cell and apoptotic inducer (Doxorubicin), C^-: Cell and snake venom fraction, Tests: Cell and virus and snake venom fraction

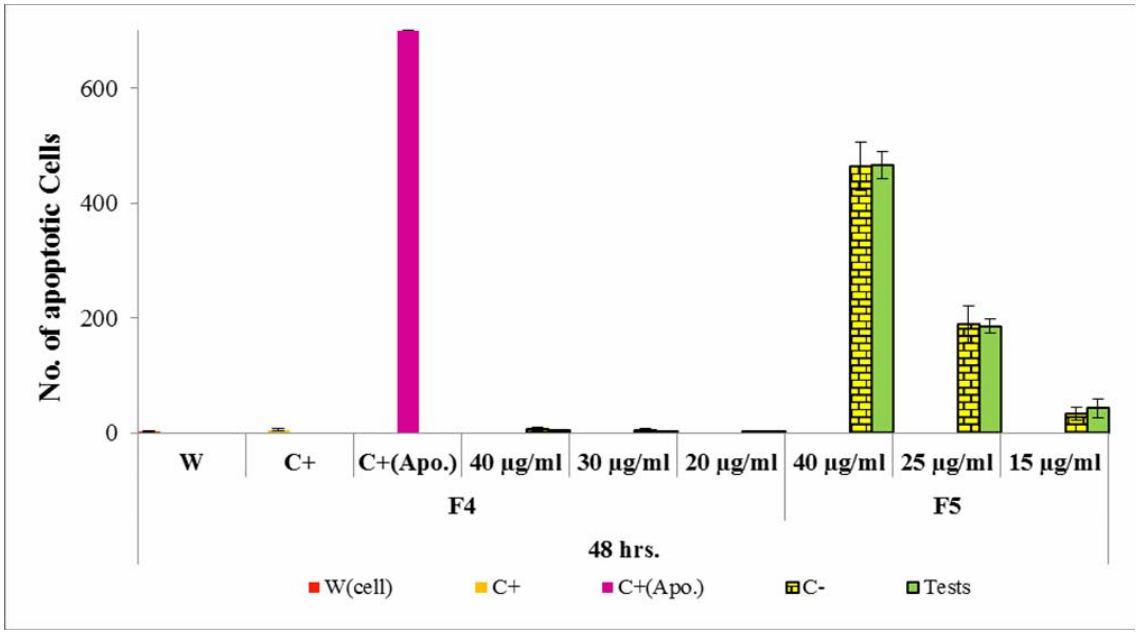

Figure 4. Evaluation of the effects of venom fractions (F4, F5) on apoptosis of Infected BHK-21 cells in 48h. W: cells without treatment, C^+: cell and virus, C^+ (APO): cell and apoptotic inducer (Doxorubicin), C^-: Cell and snake venom fraction, Tests: Cell and virus and snake venom fraction

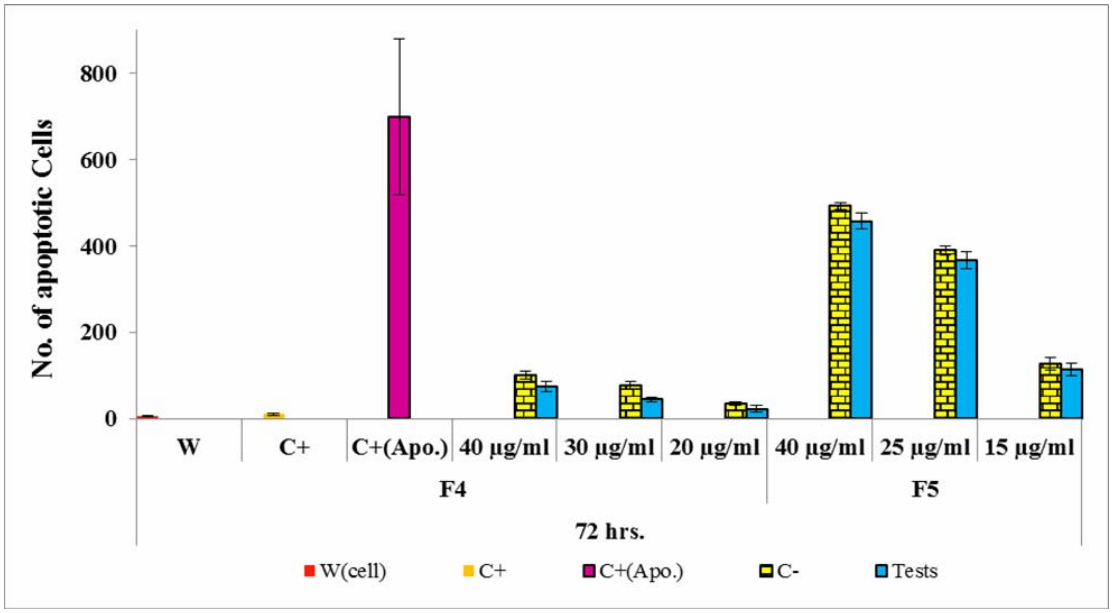

Figure 5. Evaluation of the effects of venom fractions on (F4, F5) apoptosis of Infected BHK-21 cells in 72h. W: cells without treatment, C^+: cell and virus, C^+ (APO): cell and apoptotic inducer (Doxorubicin), C^-: Cell and snake venom fraction, Tests: Cell and virus and snake venom fraction

3.3. Statistical analysis

Data were statistically analyzed using the one-way ANOVA method**.** The data were expressed as Mean± SD.

4. Discussion

In the present study, apoptosis was investigated in BHK- 21 cell lines infected by rabies virus. Snake venom contains biologically active components prone to acetylcholine receptors and ion channels on the cell surface. These compounds are able to reduce the accumulation of the virus at the nerve site, which can lead to inhibition of virus activity. One study demonstrated that snake venom from *Vipera lebetina turanica* kills apoptotic cells of neuroblastoma cells through ROS-dependent MMP disorder, and suggests that snake venom may be an anticancer agent for neuroblastoma. The apoptotic effect of snake venom on human SK-N-MC and SK-N-SH neurons was investigated. The results showed that cell isolation and apoptotic cell death were increased by snake venom (1.25-10µg/mL), but normal neurons were not affected (Alizadeh et al., 2015). The venom of Naja snake is specific to the cobra family and it consists of neurotropic molecules. There are two types of cobras called *Naja naja oxiana* and *Walterinnesia aegyptia*, belong to genus Elapidea and are found in Iran. Considering the therapeutic effects of *Naja naja oxiana* venom as well as its low side effects compared to neuro-drugs, it seems necessary to conduct a study on the therapeutic effects of this toxin (Chegeni, et al., 2015).

It has recently been shown that *Walterinnesia aegyptia* (WEV) toxin, alone or in combination with silica nanoparticles (WEV+NP), causes stunted growth and apoptosis of various cancer cell lines (Barat and Davoudi Dahaghani, 2018). Following a viral infection, the programmed cell death of cells infected with apoptosis is a cellular response, and its function is primarily to limit the spread of the virus (Fernandes et al., (2011). Omran M. A. A. and et al, investigated the *in vitro* process of cell death caused by Egyptian cobra venom on primary human embryonic kidney (293T) and mouse myoblast (C2C12) cell lines. They showed the apoptotic effects of *N. haje* venom on cell cultures. However, the outcome of the apoptosis pathway may depend on the concentration or timing of cells exposed to snake venom. In the present study, time and concentration were also influential factors in the rate of cellular apoptosis (Omran et al., 2004).

The results of a study on Iranian cobra venom showed that this toxin has anti-cancer effects that lead to cytotoxicity and lethality of Jurkat E6.1 leukemia cells, while it has minimal

toxicity effect on normal cell cells. According to the findings of this study, it was found that the anti-cancer activity and induction of apoptosis of recombinant protein is possible (Park et al., 2009). Infected neurons can be stimulated by migrating T cells directly or through an apoptotic mechanism stimulated by cytokines to be targeted by macrophages (Tu, 1977; Chegeni et al., 2015). If the microglia and activated T cells kill the infected neurons, the neuronal attack of the virus may be controlled. In contrast, if the microglia remains dormant and T cells are destroyed, the virus attack can spread (Baloul and Lafon, 2003).

The rabies virus delays apoptosis by controlling the expression of its proteins and regulates the survival of infected cells according to its metabolic needs. These mechanisms enable the rabies virus to escape from the host immune system and proliferate in nerve cells (Alizadeh et al., 2015).

Caspian cobra venom contains five fractions in gel filtration chromatography and certain concentration of fraction 5 (F5) was recognized as the best non-toxic fraction concentration on BHK-21 cells in previous study. In this study, for the first time we investigated the impact of two FPLC fractions (F4 and F5) of *Naja naja oxiana* snake venom on CVS-11 rabies virus infected BHK-21 cells. It was demonstrated that the infection of BHK-21 cells by rabies virus in the presence of Caspian cobra venom fractions are significantly decreased. The F5 fraction peptide of *Naja naja oxiana* snake venom enters cells through rabies virus AChR receptors without competing for binding to cellular receptors and will be able to reduce virus replication (Farzad et al., 2020).

In the present research, which was the first study to observe the effect of F4 and F5 fractions of Caspian cobra snake venom on induction of apoptosis on virus-infected cells. To prove it, we also used the Hoechst reagent. Allen S., et al used the Hoechst 33342 staining to detect apoptotic changes in bovine mononuclear phagocytes infected with *Mycobacterium avium* subsp. Paratuberculosis. They observed an increase in the number of apoptotic monocytes within 6 hours after infection with *M. avium* subsp, which increased at 24 and 48 hours. Our results showed apoptosis reveals several characteristic morphological changes, such as chromatin condensation and nuclear fragmentation. Doxorubicin was used as an agent to induce apoptosis and as a positive control as well. Concentrations of 3.0µg/ml of Doxorubicin solutions were used as a positive control at all three incubation times of 24, 48 and 72 hours, and all graphs showed that the number of apoptotic cells reached more than 400 cells. In the previous study we demonstrated the cytotoxic effects of different concentrations of *Naja naja oxiana* fractions (F1–F5) obtained from FPLC examined on BHK-21 cells. Rabies infected BHK-21 cells were exposed to two fractions, F4 and F5 of FPLC fractions (Farzad et al., 2020). The number of uninfected, infected, treated with Doxorubicin, treated with defined concentration of F4 and F5 fractions, infected apoptotic BHK-21 cells in the presence of different concentrations of two fractions F4 (40, 30 and 20µg/ml) and F5 (40, 25 and 15µg/ml) of *Naja naja oxiana* venom are illustrated in Figure 1, by considering the positive control.

According to our study and statistical analysis evaluation the effect of both fractions F4 and F5 at three times of incubation (24, 48 and 72 hours), the F5 fraction with non-toxic concentration (25µg/ml), had the maximum inhibiting and apoptotic effects on infected BHK-21 cells after 48 hours of incubation time.

5. Conclusion

There are two different mechanisms for cell death, necrosis and apoptosis by Cobra venom. Apoptosis caused by the cytotoxic components of cobra venom fractions activate a death program that leads to irreversible damage and apoptotic effects on BHK-21 cells. This study showed the apoptotic effect of Caspian cobra venom inhibits virus proliferation in mechanism associated with cellular apoptosis similar to *Naja haje* venom at the cellular level. The contribution of the apoptotic pathway may, however, be dependent on concentration and/or time of exposure to cobra venom (Omran et al., 2004). The F5 fraction induced morphological alterations that proceed cell death. Our results showed the chronological events leading to apoptosis. Doxorubicin was used as an inducer of cellular apoptosis. Hoechst dye was used to observe cell morphological changes. Our observations have shown that the use of *Naja naja oxiana* snake venom can cause apoptosis of

rabies-infected cells, so we can hope to use this toxin as a drug lead of an anti-rabies agent.

Conflict of Interest:

The authors declare that there is no conflict of interests regarding the publication of this article.

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Refereces

Alizadeh, L., Akbari Dana, M., Barati Dowom, P., Ghaemi, A., (2015). Immunology of Rabies Virus in the Central Nervous System. Shefayekhatam. 3(3) [in Persian].

Badr, G., Sayed, D., Maximous, D., Mohamed, A.O., Gul, M., (2014). Increased susceptibility to apoptosis and growth arrest of human breast cancer cells treated by a snake venom-loaded silica nanoparticles. Cellular Physiology and Biochemistry. 34: 1640-1651.

Baloul, L., Lafon, M., (2003). Apoptosis and rabies virus neuroinvasion. Biochimie 85, 777-788.

Barati, M., Davoudi Dahaghani., (2018). Evaluation of toxicity and anticancer activity of isolated fraction from the venom of Iranian cobra snake on acute lymphoblastic leukemia cells (Jurkat E6.1). Journal of Cell & Tissue. 8(3): 101-108

Chakrabarty, D., Sarkar, A., (2016). Cytotoxic Effects of Snake Venoms. Snake Venoms. 1-16.

Chegeni, Z.H., Oryan, S., Abadi, A.Z.M., Bakhtiarian, A., Akbari, S., Ghamami, G., Nazari, K., (2015). The Antinociceptive Effects of Iranian Cobra Snake Venom using Formalin Test. Arak Medical University Journal 18, 80-92 [In Persian].

Consales, C., Bolzan, V., (2007). Rabies review: immunopathology, clinical aspects and treatment. Journal of Venomous Animals and Toxins including Tropical Diseases. 13: 5-38.

Costal-Oliveira, F., Stransky, S., Guerra-Duarte, C., de Souza, D.L.N., Vivas-Ruiz, D.E., Yarlequé, A., Sanchez, E.F., Chávez-Olórtegui, C., Braga, V.M., (2019). L-amino acid oxidase from Bothrops atrox snake venom triggers autophagy, apoptosis and necrosis in normal human keratinocytes. Scientific reports. 9: 1-14.

Dehesa-Davila M., Alagon A.C., Possani L.D., (1995). Clinical toxicology of snakebite in Africa and the Middle East / Arabian Peninsula. In: MEIER J., WHITE J. Eds. Handbook of clinical toxicology of animal venoms and poisons. New York: CRC Press: 433-92.

El-Fiky, M., (1999). Hyperglycemic effect of a neurotoxic fraction (F3) from Naja haje venom: role of hypothalamo-pituitary adrenal axis (HPA). Journal of natural toxins. 8: 203-212.

Farzad, R., Gholami, A., Hayati Roodbari, N., Shahbazzadeh, D., (2020). The anti-rabies activity of Caspian cobra venom. Toxicon. 186: 175–181.

Fernandes, E.R., de Andrade Jr, H.F., Lancellotti, C.L.P., Quaresma, J.A.S., Demachki, S., da Costa Vasconcelos, P.F., Duarte, M.I.S., (2011). In situ apoptosis of adaptive immune cells and the cellular escape of rabies virus in CNS from patients with human rabies transmitted by Desmodus rotundus. Virus research. 156: 121-126.

Hassouna, I.A., Rahmy, T.R., (2001). Reactive astrocytic response and increased proliferating cell nuclear antigen expression in cerebral cortex of envenomed rats. Journal of Toxicology: Toxin Reviews. 20: 245-259.

Houchins, J., (2000). Immunotoxin-Induced Apoptosis. Stem Cells. 18: 384-385.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227 (5259): 680–685.

Lyone, GI., Esther, DI., (1999). Apoptosis. Stem cells. 17: 306-13.

Maristela, P., Daniela. DC., Antonio, RG., Delwood, CC., (1999). The effect of lectin from the venom of snake, Bothrops jararacussu, on tumor cell proliferation. Anticancer Res. 19: 4023-4026.

Mohamed, A., Hanna, M., (1973). The in vivo anticoagulant effects of Naja flava venom. Toxicon. 11: 419-422.

Mosmann, T., (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65 (1–2): 55–63.

Omran, M., (1997). Monitoring of the electrocardiographic disorders of rat after administration of sublethal doses of Egyptian snake (cobra) venom. J. Nat. Toxins. 6: 261-274.

Omran, M., Abdel-Nabi, I., El-Naggar, M., (1997). Serum biochemical and hormonal parameters as biomarkers for the toxic effects of Egyptian cobra (Naja haje) envenomation. Journal of Natural Toxins. 6: 69-84.

Omran, M., Fabb, S., Dickson, G., (2004). Biochemical and morphological analysis of cell death induced by Egyptian cobra (Naja haje) venom on cultured cells. Journal of Venomous Animals and Toxins including Tropical Diseases. 10: 219-241.

Omran, M.A.A., Abdel-Nabi, I.M., (1997). Changes in the arterial blood pressure, heart rate and normal ECG parameters of rat after envenomation with Egyptian cobra (Naja haje) venom. Human & experimental toxicology. 16: 327-333.

Park, M.H., Son, D.J., Kwak, D.H., Song, H.S., Oh, K.-W., Yoo, H.-S., Lee, Y.M., Song, M.J., Hong, J.T., (2009). Snake venom toxin inhibits cell growth through induction of apoptosis in neuroblastoma cells. Archives of pharmacal research. 32: 1545-1554.

Rahmy, T., Hemmaid, K., (2000). Histological and histochemical alterations in the liver following intramuscular injection with a sublethal dose of the Egyptian cobra venom. Journal of natural toxins. 9: 21-32.

Rahmy, T., Ramadan, R., Farid, T., El-Asmar, M., (1995). Renal lesions induced by cobra envenomation. J. Egypt. Ger. Soc. Zool. 17: 251-271.

Saedi, N., Amin zadeh, S., (2018). Therapeutic potential of snake venom in the treatment of cancer. Journal of Laboratory Diagnosis. 145: 29-33 [In Persian].

Takeuchi, H., Baba, M., Shigeta, S., (1991). An application of tetrazolium (MTT) colorimetric assay for the screening of anti-herpes simplex virus compounds. J. Virol Methods. 33 (1–2): 61–71.

Tu, A., (1977). Chemistry and Molecular Biology. New York: Venoms. John Wiley Sons.

Tu, A., (1991). Tissue damaging effects by snake venoms: hemorrhage and mionecrosis. Handbook of Natural Toxins: Reptile venoms and toxins.

Tu, A.T., (1991). Tissue damaging effects of snake venoms, Handbook of Natural Toxins: Reptile Venoms and Toxins. Marcel Dekker Inc. New York, p. 827.

Vural, S.A., Bozkurt, M.F., Ozkara, A., Alcigir, M.E., Ilhan, F.S., (2016). Apoptosis in natural rabies virus infection in dogs. Journal of Veterinary Research. 60: 227-231.

Wang, Y., Jing, L., Xu, K., (2002). A unique approach for high level expression and production of a recombinant cobra neurotoxin in Escherichia coli. Journal of biotechnology. 94: 235-244.

Warrell, D., (1995). Clinical toxicology of scorpion stings. Handbook of clinical toxicology of animal venoms and poisons.

Warrell, D., Greenwood, B., Davidson, N.M., Ormerod, L., Prentice, C., (1976). Necrosis, haemorrhage and complement depletion following bites by the spitting cobra (Naja nigricollis). QJM: An International Journal of Medicine. 45: 1-22.