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Original Article

Antiproliferative Effects of Wild-Type *Echovirus* in Human Lung Cancer Cells

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Abstract

Introduction: Oncolytic virotherapy is a new method of treating cancer. Various types of viruses have been shown to inhibit the growth of cancer cells due to the high presence of specific receptors on cancer cells. In this study, our aim was to investigate the potential ability of wild-type *Echovirus* to suppress the proliferation of A549 cell line as human lung cancer cells.

Materials and Methods: In this study, we assessed the effects of treating A549 cells with both wild-type *Echovirus* and doxorubicin (DXR). We focused on several key parameters including cell proliferation, reactive oxygen species (ROS) generation, lactate dehydrogenase (LDH) release rate, apoptosis percentage, as well as caspase-8 and caspase-9 activities.

Results: The cytotoxic effects of wild-type *Echovirus* were measured on A549 cell lines in all treatment groups by MTT and apoptosis assay. The lowest and highest cytotoxic effects and apoptosis percentage were associated with the 10 and 40 MOIs of *Echovirus* treated groups, respectively. Additionally, *Echovirus* significantly increased LDH and ROS generation and the activities of caspase-8 and caspase-9 compared with the control group.

Conclusions: Wild-type *Echovirus* has the potential to inhibit the proliferation of lung cancer cells and promote apoptosis through both mitochondrial and extracellular apoptotic pathways. This suggests that wild-type *Echovirus* could be considered as a possible therapy for human lung cancer in the future.

Keywords: Oncolytic Virus, *Echovirus*, Human Lung Cancer, Apoptosis Pathways

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Introduction

Lung cancer (LC) is indeed the most common form of cancer among both men and women. Its high prevalence is a cause for concern.[1] The distressing reality of a 5-year survival rate, which falls below 15%, underscores the formidable challenges confronted by individuals afflicted with LC. The standard treatment options for LC have typically included chemotherapy, surgery, and radiation. Yet, the efficacy of these treatments can be unpredictable, underscoring the pressing demand for enhanced therapeutic alternatives.[2] Unfortunately, many patients with LC develop resistance to existing medications over time, leading to disease recurrence.[3] This underscores the urgency of exploring innovative treatment modalities that target novel molecular pathways, offering a distinct paradigm for tackling the disease. Such groundbreaking therapies hold the potential to augment the overall survival rates of LC patients.[4] Oncolytic viruses (OVs) are gaining recognition as biotherapeutics capable of selectively targeting and annihilating tumor cells. By eliciting tumor-targeted immune responses within the host and inducing the lysis (destruction) of tumor cells, OVs hold the potential to eliminate tumors effectively.[5,6] Moreover, OVs can modify the tumor microenvironment (TME) by triggering the production of inflammatory cytokines and releasing tumor antigens. This alteration in the TME can enhance the uptake of tumor antigens and stimulate the maturation of antigen-presenting cells such as macrophages and dendritic cells. This combined effect holds promise for developing more effective strategies in cancer treatment.[7] Several OVs have undergone extensive investigation in clinical trials, with notable examples including the herpes simplex virus and adenoviruses. Additionally, other OVs like the Newcastle disease virus, poxviruses, and reovirus have exhibited promising anticancer effects against diverse human tumor types.[8-10] One particular OV of interest is the *Echovirus*, which belongs to the *Picornaviridae* family, specifically the *Enterovirus* genus. *Enteroviruses* are non-

enveloped, icosahedral viruses with positive-sense single-stranded RNA genomes. They typically range in diameter from approximately 25 to 30 nm.[11] In 2004, Rigvir®, which is the brand name for the ECHO-7 virus strain (*Echovirus-7*), received approval for the treatment of melanoma in Latvia. This approval highlights its recognition as a therapeutic option for this specific type of cancer. By utilizing OVs like ECHO-7, researchers and clinicians aim to harness their unique properties to selectively target and destroy cancer cells while preserving healthy cells.[12] *Echoviruses* utilize decay-accelerating factor (DAF) as their cellular receptor. DAF, a glycosylphosphatidylinositol-anchored complement regulatory protein, is commonly present on most cell surfaces and primarily functions to shield cells from immune system complement attacks.[11] Notably, cryo-electron microscopy reconstructions of *Echoviruses* complexed with DAF have unveiled unique structural characteristics.[13] It has been demonstrated through various studies that ECHO-7 can reduce the viability of certain cancer cells *in vitro*, including human melanoma, rhabdomyosarcoma, gastric adenocarcinoma, lung carcinoma, and pancreatic adenocarcinoma.[11] However, it does not exhibit the same effect on PBMCs (peripheral blood mononuclear cells), suggesting a specific anti-cancer effect.[11] In the present study, we aimed to determine the possible anti-cancer effects of wild-type *Echovirus* on human lung cancer (cell line A549).

Materials and Methods

Cell Line and Treatment of the Virus

A549 cells were obtained from the Pasteur Institute located in Tehran, Iran. These cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with the addition of 10% FBS (Fetal Bovine Serum). A549 cells were maintained at a temperature of 37 °C in a humidified environment with 5% CO_2. The wild-type *Echovirus-6* was obtained from the Applied Virology Research Center of Baqiyatallah University of Medical Sciences. Subsequently, A549 cells were infected with *Echovirus* at different MOI (Multiplicity of Infection) levels to assess its cytotoxic effects.

Evaluation of Echovirus Cytotoxic Effects

To evaluate the cytotoxicity of *Echovirus*, we conducted the MTT test. A549 cells were cultured in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 hours at 37 °C in a humid environment with 5% CO_2. After this initial incubation period, we added different MOIs (10, 20, 40) of *Echovirus*, along with DXR (10 nM),[14] to each respective well and allowed them to incubate for 4 hours. Following this incubation, we removed the medium and replaced it with fresh culture medium. As a control, three wells were maintained with DMEM. After an additional 72-hour incubation period, we added 25 µl of MTT solution (5 mg/ml in PBS) to each well and incubated the plates at 37 °C for 4 hours. Subsequently, we added 100 µl of dimethyl sulfoxide (DMSO) to dissolve the resulting purple formazan crystals. Finally, we measured the absorbance of light at 570 nm using an ELISA reader.[15]

Evaluation of Lactate Dehydrogenase (LDH) Activity

Lactate dehydrogenase (LDH) is an enzyme located in the cytosol responsible for converting lactate to pyruvate. When the plasma membrane is damaged or ruptured, LDH is released into the culture media, leading to an elevated extracellular level. Cytotoxicity was assessed using the LDH Kit from ZellBio GmbH in Germany. This kit quantifies the release of LDH, an enzyme found in the cytoplasm, from damaged cells. Cells cultivated in 96-well plates were exposed to *Echovirus* and DXR treatments. Following a 72-hour treatment period, the culture supernatant was collected and mixed with a reaction mixture. The LDH enzyme present in the supernatant catalyzed a conversion process, resulting in the reduction of a tetrazolium salt, forming a compound called Formosan. The quantity of Formosan produced was determined by measuring the absorbance at a wavelength of 492 nm.

Measurement of Reactive Oxygen Species (ROS)

Dichlorodihydrofluorescein diacetate (DCF-DA, 2.5 µM; Invitrogen, St Louis, MO, USA) was introduced to the cells, entering passively. Inside the cells, DCF-DA reacted with ROS, generating dichlorofluorescein (DCF), a highly fluorescent compound. The 1×10^6 cells/well were briefly exposed to *Echovirus* and DXR in 6-well plates for 4 hours, followed by a 72-hour incubation period. After two washes with PBS, the cells were stained with 20 µM DCFH-DA for 30 minutes and subsequently incubated. Following another PBS wash to remove any residual staining solution, the fluorescence intensities were measured and compared to the control. The treated cells were observed under a fluorescence microscope (Olympus CKX 41) at a 40X magnification.

Evaluation of Survival Rate/Apoptosis

To examine the apoptosis percentage of A549 cells in response to *Echovirus* and DXR treatment, we used acridine orange and propidium iodide stains. Initially, the isolated cells were treated with *Echovirus* and DXR in 24-well plates, and then detached using a 0.05% trypsin/EDTA solution. The cell suspension for each treatment group (*Echovirus* and DXR) was subsequently rinsed with PBS. Following the PBS rinse, a fluorescent dye containing acridine orange (10 µg/ml) and propidium iodide (10 µg/ml) added to the cellular pellet in a volume of 10 µl. The estimation of apoptotic cells (%) was then conducted using fluorescent microscopy, specifically an Olympus CKX41 microscope. An upgraded Neubauer rhodium hemocytometer was used for this assessment.

Determination of Caspase 8/9 Activity

The researchers used caspase colorimetric assay kits (Biovision, USA) to detect caspase activation. These kits are specifically designed to measure the cleavage of a synthetic colorimetric substrate. To evaluate the activity of caspase-8 and caspase-9, A549 cells were treated with *Echovirus* for 4 hours. After 72 hours, the cells were lysed using the lysis buffer provided in the assay kits. The lysates were then subjected to centrifugation, and the resulting supernatants were collected for further analysis. To determine the caspase-8 and caspase-9 activities, equal amounts of proteins from each sample were mixed with the synthetic colorimetric substrates and incubated at 37 °C for 2 hours. The resulting reaction mixtures were then measured at a wavelength of 405 nm using a microplate reader. To calculate the fold increase in caspase-8 and caspase-9 activities, the data obtained from the treatment samples were compared to the data obtained from the control samples. BSA (bovine serum albumin) was used as the standard to ensure accurate measurement and comparison of protein amounts in each sample.

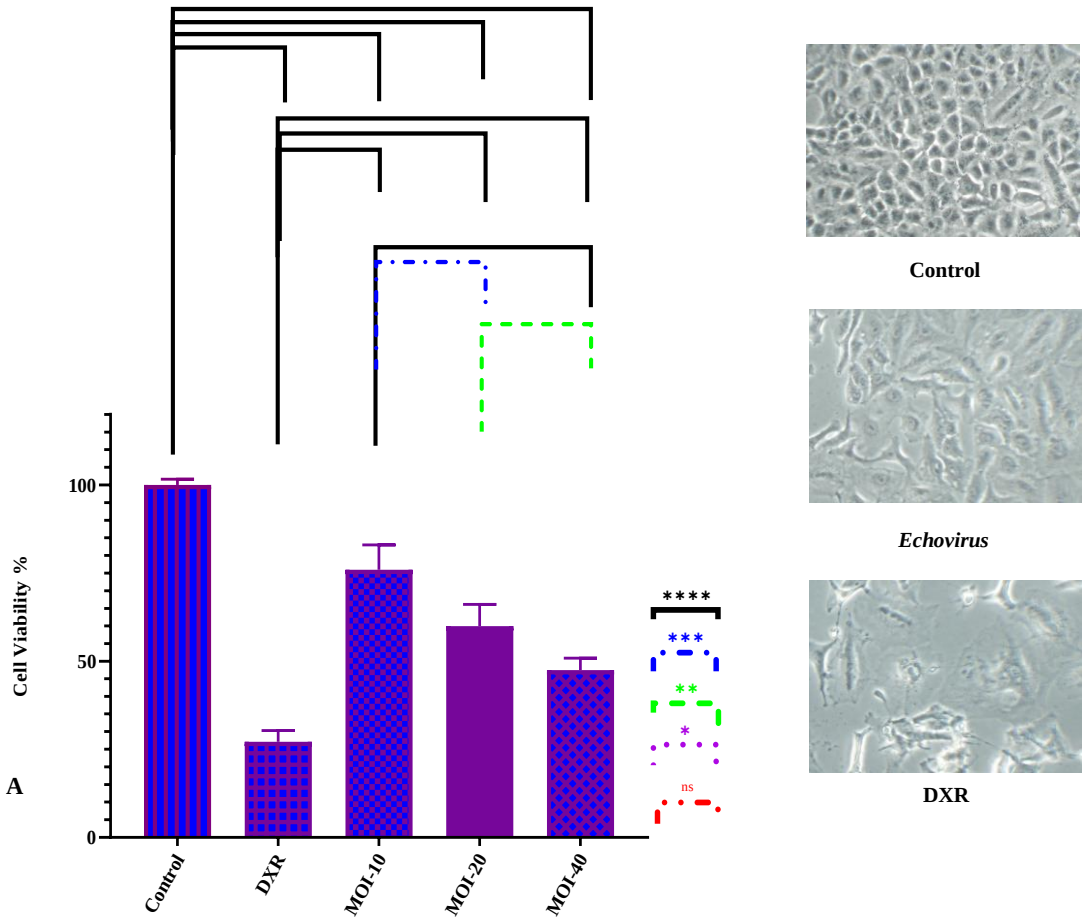
Statistical Analysis

To analyze the data, we used one-way ANOVA with Tukey's test technique. The significance threshold was set at $p<0.05$, meaning that if the probability (p-value) of obtaining the observed results by chance was less than 0.05, the differences between the groups were considered statistically significant. The graphs representing the data were created using Graph-Pad Prism 6.07 software. In the graphs, the mean values were plotted along with the standard error of the mean (SEM) to represent the variability of the data within each group.

Results

Cytotoxic Effects of Echovirus on A549 Cell Line

The cytotoxic effects of *Echovirus* on the A549 cell line were evaluated through MTT and apoptosis assays. In the MTT assay, A549 cells were treated with *Echovirus* at varying MOIs. As positive controls, A549 cells treated with DXR were employed, while untreated cells served as the negative control. Our findings demonstrated that both the *Echovirus*-treated group and the DXR-treated group exhibited significantly higher cytotoxic effects compared to the negative control group. When assessing the different MOIs of *Echovirus* treatment, the lowest and highest cell viability percentages were observed in the 40 MOI and the 10 MOI treated groups, respectively, compared to the control group (Figure 1A). Furthermore, in the DXR-treated group, the cell viability percentage indicated a significant reduction compared



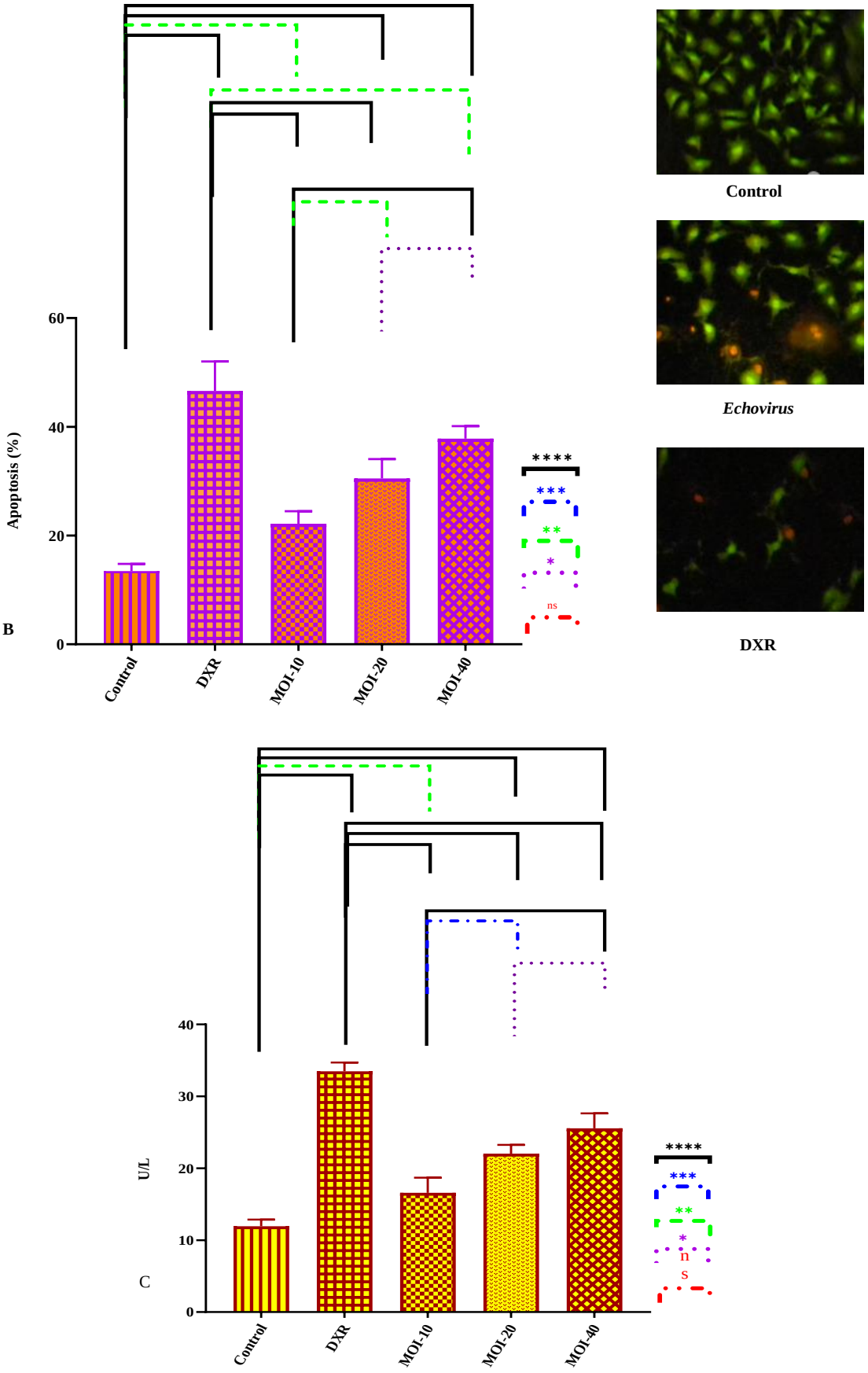


Figure 1. The study measured cell viability (A), apoptosis percentage (B), and LDH release (C) of the A549 cell line after treatment with different multiplicities of infection (MOIs) of *Echovirus* and DXR. The results were compared to the control group. Statistical significance was indicated using asterisks: (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$). The data is presented as mean ± standard deviation (SD).

to the control group. The results of the apoptosis test supported the findings of the MTT assay, revealing that both *Echovirus* and DXR treatments led to an increased percentage of apoptosis in the A549 treatment groups when contrasted with the control group. When examining the different MOIs of *Echovirus* treatment, the lowest and highest percentages of apoptosis were observed in the 10 MOI and the 40 MOI groups, respectively, compared to the control group (Figure 1B). Additionally, in the DXR-treated group, the percentage of apoptosis was significantly increased compared to the control group. Moreover, we compared the results of the MTT and apoptosis assays with the LDH release assay to assess the practicality of gauging the cytotoxicity of *Echovirus* in the A549 cell line. Our findings revealed that both *Echovirus* at all MOIs and DXR significantly elevated the levels of LDH release from A549 cells. This suggests that both treatments induce cytotoxicity in the cells. The lowest and highest percentages of LDH release were observed in the 10 MOI and the 40 MOI of *Echovirus*-treated groups, respectively, in comparison to the control group (Figure 1C). These results demonstrate that both *Echovirus* and DXR induce cytotoxicity, as evidenced by increased LDH release in the A549 cell line, providing complementary evidence to the MTT and apoptosis assays.

Fluorometric Assay for Detection of ROS

In the DCFH-DA test, A549 cells were treated with DXR as a positive control, while untreated cells served as the negative control. Across all MOIs of *Echovirus* and DXR, the percentage of DCF-positive cells demonstrated a significant increase compared to the negative control group. This indicates an elevated generation of reactive oxygen species (ROS) in response to both *Echovirus* and DXR treatments. The lowest and highest percentages of ROS generation were observed in the 10 MOI and 40 MOI of *Echovirus*-treated groups, respectively, compared to the control group (Figure 2). These results suggest that both *Echovirus* and DXR induce the generation of ROS in A549 cells, indicating oxidative stress. The elevated ROS levels observed provide further evidence of the cytotoxic effects of these treatments.

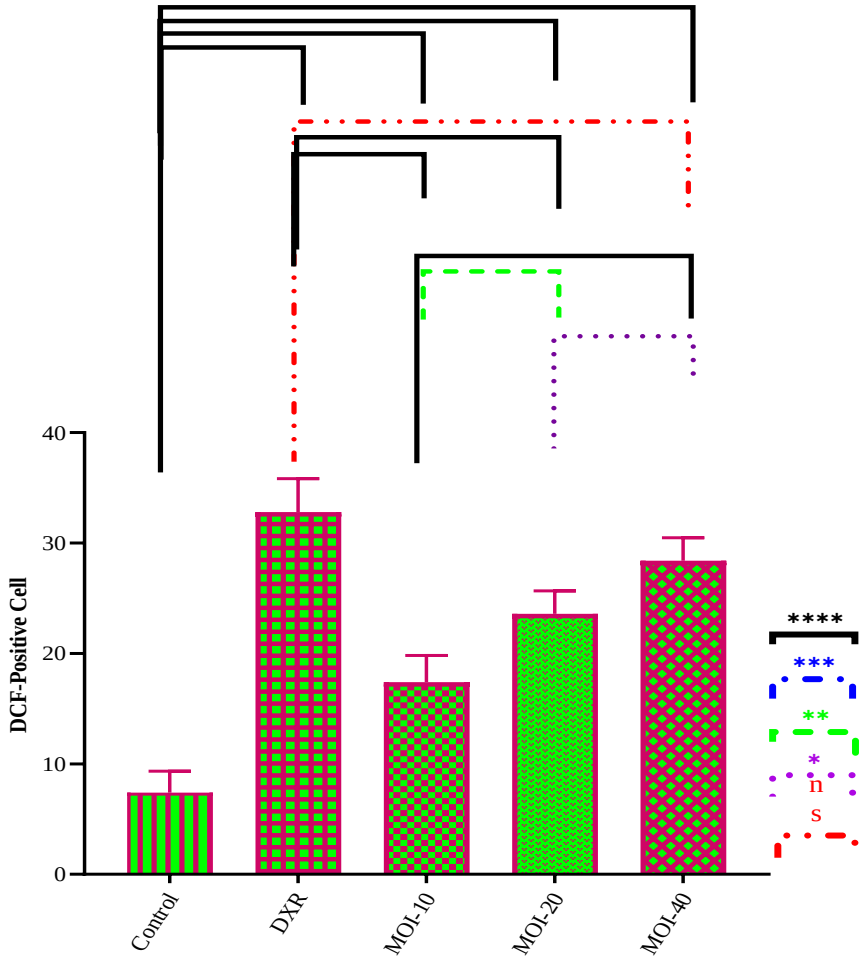


Figure 2. The study measured the percentage of ROS generation in the A549 cell line after treatment with different MOIs of *Echovirus* and DXR. The results were compared to the control group, with statistical significance indicated using asterisks: (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$). The data is presented as mean ± standard deviation (SD).

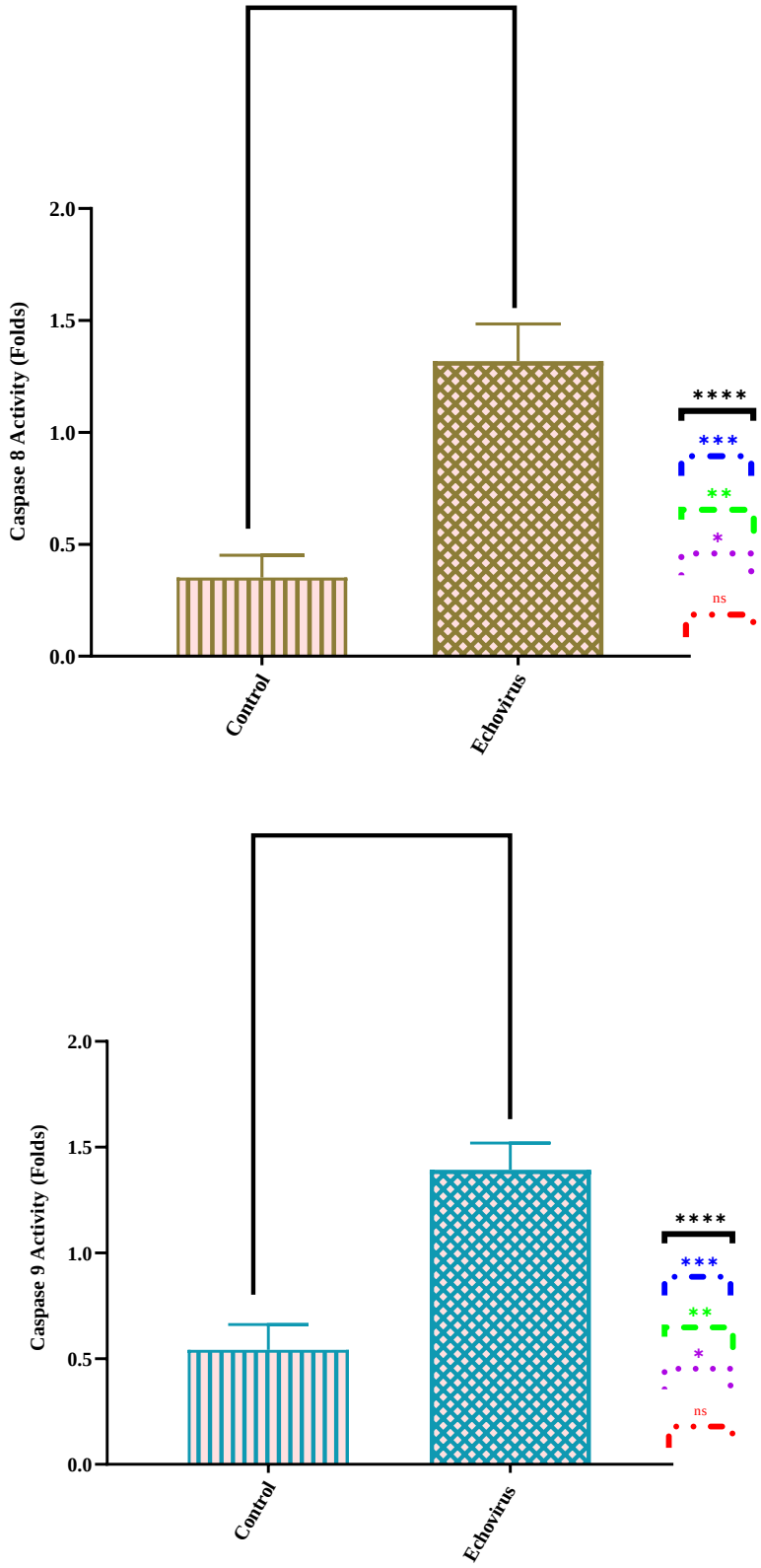


Figure 3. The study examined the activity of caspase 8 and 9 in the A549 cell line after treating it with different MOIs of Echovirus. The results were compared to the control group, and statistical significance was indicated using asterisks: (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$). The data is presented as mean ± standard deviation (SD).

Evaluation of Caspase Activity

We examined the activation of effector caspases following treatment with *Echovirus* and DXR. The results demonstrated a significant increase in the activity of caspase-8 and 9 in A549 cells treated with *Echovirus*, compared to the negative control group (Figure 3).

Discussion

Our study aimed to investigate the potential antiproliferative effects of *Echovirus* on human lung cancer using the A549 cell line. To assess cytotoxicity, we conducted MTT tests at various MOIs. The results demonstrated significant cytotoxic effects on the A549 cell line in all treatment groups. Following *Echovirus* treatment, cell viability was highest in the MOI 10 group and lowest in the MOI 40 group. Additionally, we examined LDH release, ROS generation, and apoptosis percentage to further comprehend the mechanism of action. Our findings indicated that *Echovirus* increased all three parameters in the A549 cell line, signifying its role in inducing cell death. The elevated apoptosis percentage suggests that *Echovirus* promotes programmed cell death. Furthermore, the results suggest that *Echovirus* activates apoptosis through both intracellular and extracellular pathways. Overall, our study demonstrates the antiproliferative effects of *Echovirus* on human lung cancer cells, as evidenced by reduced cell viability, increased LDH release, ROS generation, and induction of apoptosis through multiple pathways. It is noteworthy that various human enteroviruses have shown potential in clinical trials for treating various types of cancer. One example is CAVATAK®, a native picornavirus-based oncolytic virotherapy agent derived from coxsackievirus A21 (CVA21), which has been cell-adapted. CAVATAK® exhibits increased affinity for a cell surface receptor called decay-accelerating factor (DAF) compared to native CVA21.[16] Due to this enhanced affinity, CAVATAK® is capable of infecting multiple cancer types, resulting in cytolytic outcomes. This development highlights the potential of utilizing oncolytic virotherapy as a treatment strategy for cancer, using viruses that selectively target and destroy cancer cells. Further research and clinical trials are needed to fully explore the effectiveness and safety of these promising viral-based approaches in cancer therapy.[16] An alternative human picornavirus, *Echovirus* 7 (E7), has also served as a foundational virus in the development of a virus formulation for an anti-cancer drug known as Rigvir®. Rigvir® is an enterovirus used in cell-based cancer treatment called oncolytic virotherapy, originating from an *Echovirus*-7 sample. Although it is claimed that Rigvir® induces a destructive infection in various cancer cell types, there is limited molecular evidence supporting its potential for destroying cancer cells and selectively targeting them.[12] Previously, studies have demonstrated that Rigvir® is capable of decreasing the viability of human melanoma, rhabdomyosarcoma, gastric adenocarcinoma, lung carcinoma, and pancreas adenocarcinoma cells.[11] Studies have demonstrated that both the non-structural and structural regions (which encode proteins) of enteroviruses play a role in their ability to infect cells.[17] Enteroviruses require attachment to cellular surface antigens and subsequent penetration to initiate infection in host cells. Initially, the virus's surface antigen protein binds to specific receptor proteins present on the target host cells' surface.[18] The entry of the virus into the host cell and the transfer of its genetic material into the cell's cytoplasm involve interactions between viral and host cell proteins. For enveloped viruses, viral membrane fusion facilitates cell entry, while non-enveloped viruses utilize endocytosis for this purpose.[19] Specific viruses can target surface antigens expressed by cancer cells. Among these surface antigens, CD55, also known as decay accelerating factor or DAF, has been identified as a key receptor for the entry and infection of *Echovirus*-7. However, it's worth noting that other factors, such as β-2 macroglobulin, may also play a role in this process.[20] Cancer cells employ various defense mechanisms against the immune system, including the expression of membrane complement regulatory proteins. The expression of CD35, CD46, CD55, and CD59 inhibits the complement cascade, thereby blocking immune responses against cancer cells.[21] In normal tissues, membrane complement regulatory proteins, including CD35, CD46, CD55, and CD59, function to protect against accidental damage from an activated complement system. However, in cancer cells, these same proteins serve to shield the cells from recognition and attack by the immune system. Cancer cells may exhibit higher levels of these proteins and viral entry receptors, such as CD55, compared to normal cells.[22] This differential expression may create an apparent preference or tropism for viral infection in cancer cells. Once OVs enter tumor cells, they replicate within these cells, eventually inducing cell lysis, leading to the release of new viral progeny. These newly generated viruses can then invade and destroy neighboring cancer cells. The amplification of the virus within the tumor plays a crucial role in controlling the tumor by facilitating the spread of the virus from cell to cell.[23] Furthermore, the viruses released from lysed tumor cells can travel through the circulatory system, enabling them to reach and infect distant tumors. OVs often require a defect or impairment in the innate immune response to infect and replicate successfully. This defect is typically found in tumor cells but not in healthy cells.[24] Alterations in transcriptional and cell signaling pathways can also contribute to the increased susceptibility of cancer cells to OVs. For instance, cancer cells may exhibit increased expression of B-cell lymphoma-extra-large (Bcl-xL), which can render them more susceptible to viral infection. Additionally, activation of mitogen-activated protein kinases (MAPK) signaling pathways in cancer cells can also make them more susceptible to oncolytic viral therapy.[25] Besides the direct killing of tumor cells, OV infection and the subsequent cell lysis can trigger the release of danger-associated molecular patterns (DAMPs). These DAMPs contribute to the initiation of a long-lasting adaptive antitumor immune response.[26] In fact, extensive efforts have been made to develop OVs that carry transgenes designed to

induce an immunogenic cell death (ICD). The purpose of this strategy is to provoke the immune system to recognize and target tumor cells.[27] As viral replication and tumor lysis progress, antigen-presenting cells (APCs) produce cytokines, which further recruit other immune cells. The ultimate objective of this process of immune priming is to activate T lymphocytes specifically against tumor antigens, and thereby establishing an adaptive immune response to combat the tumor.[28] Studies have shown that A549 cells express high levels of CD55, making them susceptible to *Echovirus*-7 infection. Consequently, *Echovirus*-7 selectively targets tumor cells with a low-pathogenic virus, leading to tumor cell lysis through the cytopathic effects of the virus. This process also triggers an anti-tumor immune response, involving the action of immune cells to destroy tumors.[29] In addition to apoptosis, OV infection can induce different types of cell death, including autophagy, necroptosis, and pyroptosis. Understanding the patterns of cell death in tumor cells caused by viral infection is crucial for developing therapeutic strategies to sensitize tumor cells to OV therapy. This knowledge can help enhance the effectiveness of OV-based treatments.[30] Apoptosis, as a form of programmed cell death (PCD), is a non-inflammatory process triggered by specific signaling proteins in response to a death signal. It involves a cascade of events that result in morphological changes, such as cell shrinkage, chromatin condensation, and plasma membrane blabbing.[31] There are two main pathways leading to apoptosis: the intrinsic mitochondrial pathway and the extrinsic pathway, also known as the death receptor pathway. Although distinct, these pathways ultimately converge. Caspases, which are a family of cysteine aspartyl-specific proteases, play crucial roles in both the intrinsic and extrinsic pathways of apoptosis. They are involved in executing the cellular changes necessary for apoptosis to occur. In the extrinsic pathway, caspase-8 plays a pivotal role in mediating apoptosis. On the other hand, the intrinsic pathway can be initiated through caspase-9. In both pathways, the downstream executioner proteins caspase-3 and caspase-7 are cleaved, leading to the activation of apoptosis.[32] Numerous studies have demonstrated that OVs can induce apoptosis through various mechanisms, either internally or externally, or even a combination of both. Some examples of viruses known to induce death receptor-dependent apoptosis include the human immunodeficiency virus (HIV), measles virus, influenza A virus, Sindbis virus, reovirus, lyssavirus, and hepatitis C virus.[33] Several viruses have been observed to induce the relocalization of proapoptotic mitochondrial proteins from the mitochondria to the cytosol. Notable examples include human immunodeficiency virus (HIV), influenza A virus, herpes simplex virus type 1 and 8, hepatitis B virus, reovirus, and West Nile virus.[34] In a previous study, it was demonstrated that EV71 (Enterovirus 71) infection triggers apoptosis through the mitochondrial pathway. This pathway involves the release of cytochrome c from the mitochondria and the cleavage of caspase 9, which can be observed in multiple cell lines.[35] It's fascinating how viruses can manipulate host cellular processes to induce apoptosis through different pathways. A subsequent investigation disclosed that the EV71 2B protein can bring about structural alterations in the pro-apoptotic protein Bax, which then initiates the activation of the mitochondrial cell death pathway.[36] The findings from our study indicated that *Echovirus-6* induces apoptosis through both intrinsic and extrinsic pathways, as demonstrated by the measurement of caspase activity. Through biochemical experiments, it was observed that dose-dependent exposure to the *Echovirus* resulted in heightened production of lactate dehydrogenase and reactive oxygen species. *In vitro* experiments conducted by Tiligaz et al.[11] confirmed our outcomes, revealing that Rigvir® enhanced the survival rate of various cell lines. Specifically, the *Echovirus* reduced cell viability by 67% to 100% in the FM-9, RD, AGS, A549, HDFa, HPAF-II, and MSC cell lines. Among the cell lines tested, HaCaT cells exhibited only a slight response to Rigvir®, while MCF7, Sk-Mel-28, and PBMC cells showed no significant impact from the drug. *In vitro* studies demonstrated that Rigvir® reduces the viability of pancreatic adenocarcinoma cells but does not affect PBMC cell viability.[11] Additionally, six colon cancer cell lines, including LoVo, CaCo-2, SW480, SW620, T84, and HT29, were tested with human *Echoviruses* 12, 15, 17, 26, and 29. *Echoviruses* 12, 17, 26, and 29 exhibited high cytolytic activity and demonstrated rapid replication in nearly all tested cell lines. Furthermore, it was discovered that *echoviruses* 12, 17, and 26 readily infect spheroids and may possess oncolytic potential against colon cancer when evaluated in spheroids.[37] *Echovirus 1* (EV1) has demonstrated lytic infection in all eight human ovarian cancer cell lines, including OVHS-1, 2008, OVCA-429, DOV13, JAM, OVCAR-3, IGROV-1, and OAW-42. However, it does not infect human peripheral blood mononuclear cells (PBMCs) or HOSE, which is an immortalized normal ovarian surface epithelial cell line.[38] Based on the mentioned studies, it can be concluded that *Echovirus*es selectively enters cancer cells by utilizing receptors that are more highly expressed on the surface of cancer cells. Furthermore, it has been observed that *Echoviruses* increase the production of ROS, enhance the release of LDH, and induces apoptosis through both internal and external pathways. These mechanisms collectively contribute to the reduction of growth in lung cancer cells.

Conclusion

Tumor immunotherapy is increasingly relying on oncolytic viruses OVs due to their strong resistance and ability to activate the immune function. It appears that the oncolytic *Echovirus*-6 wild-type strain could be justified for cancer

treatment. The induction of apoptosis in the lung cancer cell line A549 through mitochondrial and extracellular membrane pathways by *Echovirus*-6 indicates that it could be a viable candidate for lung cancer treatment.

Authors' Contributions

RR, RK, MF, RD, AB, MT, MS, AS, HEGG: developed the theoretical formalism, performed the analytic calculations and performed the numerical simulations. Authors contributed to the final version of the manuscript. HEGG: supervised the project.

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Ethical Approval

The study protocol was reviewed and approved by the ethics committee of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1399.388).

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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