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Evaluation of the Effects of *Newcastle Disease Virus* as an Oncolytic Virus on the Expression of Apoptosis-related Genes in TC-1 Cell Line

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ABSTRACT

TC-1 is a recognized cancer cell line derived from lung epithelial cells that have been altered using the oncogenic E6 and E7 genes of human papillomavirus (HPV). These TC-1 cells are frequently utilized in preclinical research focused on lung cancer and HPV-associated tumors. The incidence of lung cancer and HPV-related cancers is significantly increasing. Drug resistance and the lack of selectivity in current treatments reduce their effectiveness. Researchers are seeking new therapeutic methods, including targeted therapies, immunotherapy, and oncolytic virus and bacterial therapies, to improve treatment outcomes and decrease mortality associated with these diseases. In this context, the present original study aimed to evaluate the potency of wild-type Newcastle disease virus (NDV-WTS) on lactate dehydrogenase (LDH) secretion and the induction of apoptosis in TC-1 cells.

In this experimental study, the TC-1 cell lines were cultured under laboratory conditions. Subsequently, they were treated with different multiplicities of infection (MOIs) of NDV-WTS (1, 2, and 4). Finally, the oncolytic effects of the virus were evaluated using laboratory assays, including MTT (cell viability), reactive oxygen species (ROS), LDH, survival rates, and the activities of Caspases 8 and 9.

The results indicated that NDV-WTS significantly decreased cell viability while increasing apoptosis, ROS levels, LDH release, and Caspase 8 and 9 activities compared to the control group. Molecular analyses further revealed that treatment of TC-1 cells with NDV significantly increased the expression of Bax, Casp8, and Casp9, while significantly decreasing Bcl2 expression relative to the control group.

NDV-WTS demonstrated remarkable efficacy in treating lung cancer and HPV-associated tumors. Based on the results of the present study, the use of Newcastle disease virus in the treatment of lung cancer and HPV-associated tumors may be beneficial, which requires further studies and clinical trials.

Keywords: Apoptosis; Gene expression; Lung epithelial cell; *Newcastle disease virus*; Tumors linked to HPV

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INTRODUCTION

TC-1 is a well-known cancer cell line that comes from lung epithelial cells modified with the oncogenic E6 and E7 genes of human papillomavirus (HPV). Researchers often use TC-1 cells in preclinical investigations related to lung cancer and HPV-related tumors. Lung cancer and HPV-related cancers are significantly on the rise, raising considerable concerns within both the medical community and the general public.[1] One of the primary challenges in treating these types of cancers is drug resistance, which arises from the frequent use of medications and their lack of selectivity. This resistance diminishes the effectiveness of treatments, thereby putting patients at greater risk. Researchers are exploring new therapeutic methods, including targeted therapies and immunotherapy. These approaches have the potential to improve treatment outcomes and reduce mortality rates associated with these diseases. With scientific advancements, there is increasing hope for better control and treatment of these cancers.[2] Since all the mentioned cancer treatments have low effectiveness and do not have selective inhibitory effects between healthy and cancer cells,[2] the use of oncolytic viruses (OVs) can be considered as a useful treatment strategy with a selective function approach. A key feature of cancer cells that makes them susceptible to selection by OVs is a defect in the production of antiviral products, including the interferon pathway. Under these conditions, OVs have maximum replication power in the cancer cells, ultimately leading to their destruction.[3] In the field of OVs, two effective approaches include the use of OVs in non- pathogenic hosts and genetically engineered viruses carrying therapeutic genes for cancer treatment.[4] On October 27, 2015, the FDA approved Talimogene laherparepvec (T-VEC) for melanoma patients with non-surgically removable lesions. Another OV, ONYX-015, is approved in China for head and neck cancer. Various oncolytic viruses, including oHSVs, Pexa-Vec, Measles Virus, Cavatak, PVS-RIPO, Reolysin, ParvOryx, VSV, and Newcastle disease virus (NDV), have shown anticancer effects in studies.[5] NDV, known as Avian Paramyxovirus Type 1 (APMV-1), belongs to the Paramyxoviridae family and serves as the etiologic agent of Newcastle disease in poultry. This virus acts as an oncolytic, selectively targeting cancer cells and exhibiting strong immunogenicity and broad antitumor activity. NDV has been associated with reduced tumor growth and increased survival in patients, proving effective in treating human tumors such as hepatocellular carcinoma and glioblastoma.[6] Dormant tumor cells, including stem cells, are often resistant to chemotherapy and radiotherapy; however, they may be vulnerable to NDV. Oncolytic NDV is capable of significant replication within human cancer cells, increasing by up to 10 000 times. This virus acts by binding to cancer cells and entering through receptor-mediated endocytosis. NDV exhibits various anticancer effects, including enhancing the host immune response and activating apoptosis pathways in cancer cells.[7] Wild-type NDV displays stronger oncolytic effects compared to other strains due to its unique genetic features, enhanced replication in cancer cells, and superior immune response stimulation. Previous studies support these claims, highlighting its potential for effective cancer treatment and resistance to conventional therapies.[8] Several clinical trials have been conducted utilizing wild-type NDV strains in cancer patients, showing minimal side effects. Oncolytic NDV is recognized as a promising approach for cancer treatment and offers advantages in safety and cost compared to recombinant strains.[9] Viral oncolytic therapy faces limitations, including immune clearance that reduces efficacy, tumor-induced immunosuppression hindering viral replication, and inflammatory responses causing side effects. Additionally, tumor heterogeneity can lead to variable responses, while limited viral persistence and safety concerns regarding off-target effects further challenge the effectiveness of this treatment approach.[10,11] The aim of the present study was to evaluate the effects of the NDV wild-type strain (NDV-WTS) on the expression of apoptosis-related genes and lactate dehydrogenase (LDH) secretion in lung cancer and HPV-related cancers.

MATERIALS AND METHODS

Experimental Design

TC-1 cells were obtained from the Virology Research Center in Tehran, Iran, and cultured in DMEM (Gibco, UK) with 10% fetal bovine serum (FBS) under 5% CO_2 at 37°C. The NDV-WTS (a specific 50% tissue culture infectious dose [$TCID_{50}$]) was sourced from the Applied Virology Research Center at Baqiyatallah Medical Science University.

MTT Assay

The cytotoxic effects of NDV-WTS were assessed using the Cell Proliferation Assay Kit (MTT) from Sigma, USA. TC-1 cells were plated at a density of 3×10^4 and incubated for 24 hours. They were then treated with NDV-WTS at multiplicities of infection (MOIs) of 1, 2, and 4 for 1 hour. After discarding the medium, DMEM was added, and doxorubicin was used as a positive control at 800 nM. After 72 hours, 25 µL of MTT solution (5 mg/mL in PBS) was added and incubated for 4 hours. DMSO was then added to dissolve the formazan crystals, and absorbance was measured at 570 nm.

LDH Release Measurement

To evaluate cell lysis, the secretion of LDH into the culture medium was assessed using the LDH assay kit according to the manufacturer's protocol (Kiazist, Iran). TC-1 cells were seeded in 96-well plates at a density of 3×10^4 cells per 100 µL, with treatment procedures consistent with the MTT assay. After a 72-hour incubation, the plates were centrifuged for 10 minutes at 250*g*. Next, 100 µL of supernatant from each well was transferred to a new plate, and 100 µL of LDH test solution was added. Following a 30-minute incubation at room temperature, absorbance was measured at 570 nm, and the results were analyzed.

ROS Production Assay

Reactive oxygen species (ROS) levels were measured using a commercial kit (Kiazist, Iran). TC-1 cells were plated in 6-well plates at a density of 5×10^5 cells per well, following the MTT assay protocol. After 72 hours, the supernatant was discarded, and fluorescence was assessed with a microplate fluorimeter at 485/528 nm.

Apoptosis Assay

The percentage of apoptotic TC-1 cells was determined using propidium iodide and acridine orange against NDV-WTS at MOIs of 1, 2, and 4, as well as with doxorubicin treatment at 800 nM, based on the methodology by Esmaeili Govarchin Ghaleh et al (2019).[12] Cells isolated from 24-well plates were treated with a 0.05% trypsin/EDTA solution. After rinsing the cell suspension with PBS, a fluorescent dye mixture (10 µL) containing propidium iodide (10 µg/mL) and acridine orange (10 µg/mL) was added to the pellet. Apoptotic cell percentages were assessed using fluorescent microscopy (Olympus CKX41) and a Neubauer hemocytometer.

Caspase 8 and 9 Activity Assays

Caspase 8 and 9 activities were measured using colorimetric assay kits (Sigma-Aldrich) after treating TC-1 cells with NDV-WTS and Doxorubicin (800 nM), following manufacturer guidelines.[12]

RNA Extraction

To extract RNA, 1 mL of RNX-Plus solution (Sinaclon) was added to 10^6 cells and homogenized. The solution was transferred to a 2 mL microtube, vortexed for 5 seconds, and incubated at room temperature for 5 minutes. Then, 200 µL of chloroform was added and shaken well for 15 seconds. The microtube was incubated on ice for 5 minutes and then centrifuged at 12 000 rpm for 15 minutes at 4°C. The aqueous phase was carefully transferred to a new RNase-free microtube, and an equal volume of isopropanol was added. After gentle mixing and a 15-minute incubation on ice, it was centrifuged again. The supernatant was discarded, and 1 mL of 75% ethanol was added before centrifuging at 4°C for 8 minutes. After discarding the supernatant, the pellet was dried, dissolved in 50 µL of DEPC-treated water, and placed in a 55-60°C water bath for 10 minutes. The concentration and purity of the RNA were assessed using a Nanodrop spectrophotometer.

cDNA Synthesis

The cDNA synthesis was performed using the Sinaclon cDNA synthesis kit (Sinaclon, Iran). In a 0.2 mL microtube, 1 µg of RNA was combined with 1 µL of oligo (dT), 1 µL of dNTP mix (10 mM), 4 µL of Buffer M-MuLV (5X), and 1 µL (200 U) of M-MuLV Reverse Transcriptase, making a final volume of 20 µL with DEPC-treated water. The mixture was incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes, and finally at 70°C for 15 minutes. The synthesized cDNA's quality and quantity were evaluated using a Nanodrop spectrophotometer and stored at -20°C for later use.

Real-Time PCR

For real-time PCR, Prime Q-Master Mix with SYBR Green I (Parstous, Iran) was utilized. The amplified genes included *Casp8*, *Casp9*, *Bax*, and *Bcl2*, with *Gapdh* as the internal control (Table 1, Supplementary). The protocol involved an initial cycle at 95°C for 15 minutes, followed by 40 cycles of 15 seconds at 95°C, 20 seconds at the annealing temperature, and 15 seconds at 72°C.

Statistical Analysis

Data analysis was performed using SPSS-18 software, presenting information as mean ± standard deviation. Normality of quantitative variables was confirmed with the Kolmogorov-Smirnov test. One-way ANOVA and Tukey's test were employed for data analysis. Figures were plotted using GraphPad Prism version 8, with a significance level set at $p<0.05$ for all evaluations.

RESULTS

Cell Viability and Cytotoxicity Assays

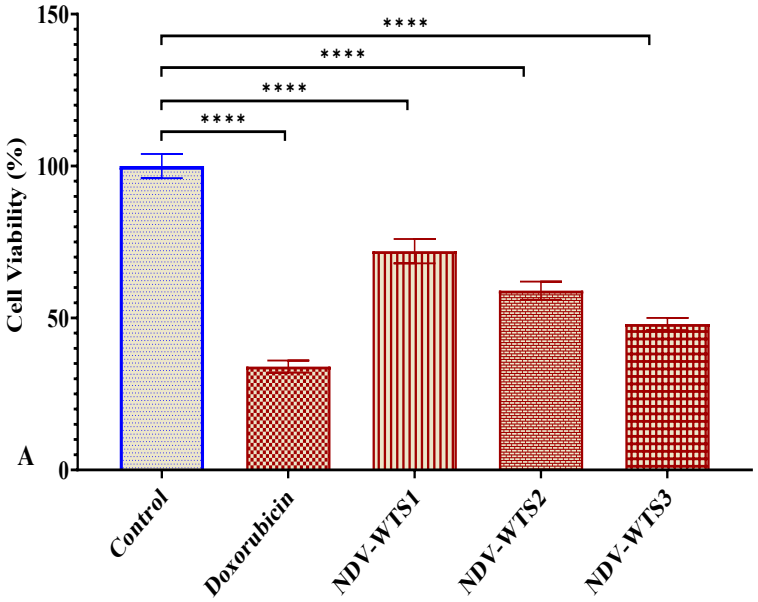
To assess the effects of NDV-WTS on TC-1 cell viability, MTT and apoptosis assays were conducted. Figure 1A and 1B indicate that all treatment groups significantly reduced cell viability and increased apoptosis compared to the control group ($p<0.05$). The Doxorubicin-treated group, as the positive control, exhibited the lowest cell viability and highest apoptosis percentage ($p<0.0001$). Significant differences were noted between NDV-WTS and Doxorubicin regarding cell viability and apoptosis ($p<0.0001$). Additionally, NDV-WTS demonstrated a concentration-dependent decrease in cell viability and an increase in apoptosis compared to the control group ($p<0.0001$).

LDH Release

LDH is released during apoptosis and necrosis, serving as a marker for cellular damage. Elevated LDH levels indicate cell death and tissue injury in various pathological conditions. Figure 2 shows that LDH release significantly increased in all treatment groups compared to the control group ($p<0.05$). The Doxorubicin-treated group exhibited the highest LDH levels relative to the control ($p<0.0001$). Furthermore, all NDV-WTS treated groups showed a significant increase in LDH release compared to the control ($p<0.0001$). Results indicate that higher virus concentrations corresponded with increased lactate dehydrogenase release ($p<0.0001$).

ROS Production

ROS are highly reactive molecules essential for cellular signaling and homeostasis, but excessive production can cause oxidative stress, promoting cancer progression and cell damage. Figure 3 illustrates that ROS levels significantly increased in all treatment groups, except NDV-WTS1, compared to the control ($p<0.05$). The Doxorubicin group exhibited the highest ROS levels among treatments ($p<0.0001$). All NDV-WTS treated groups, except NDV-WTS1, showed a significant increase compared to the control ($p<0.0001$). Results indicate that as NDV-WTS concentration rises from NDV-WTS1 to NDV-WTS2, ROS production increases ($p<0.05$), but the increase from NDV-WTS2 to NDV-WTS3 is not significant ($p>0.05$).



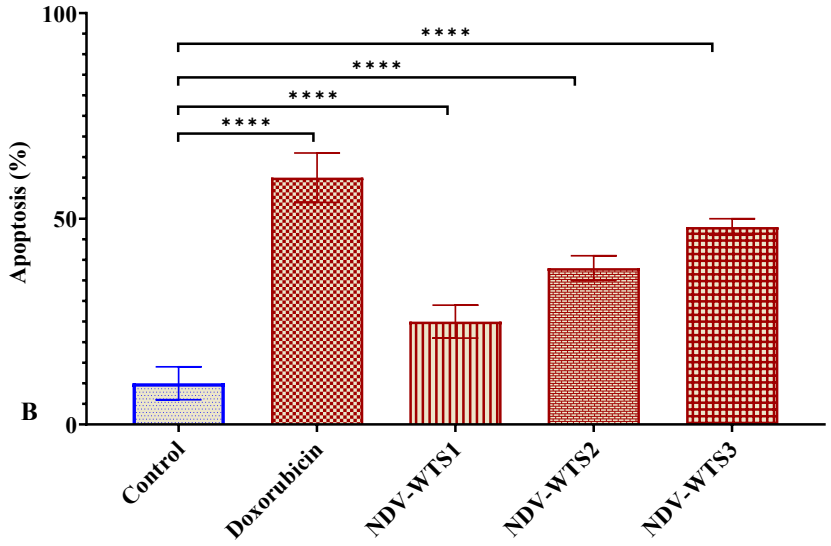


Figure 1. Effects of NDV-WTS on TC-1 cell viability and apoptosis. A. Cell viability of TC-1 cells under the influence of different treatments, measured by MTT assay. B. Percentage of apoptotic TC-1 cells after various treatments. The changes demonstrate the effects of the treatments and can aid in better understanding the effectiveness of therapeutic methods. Data are plotted as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

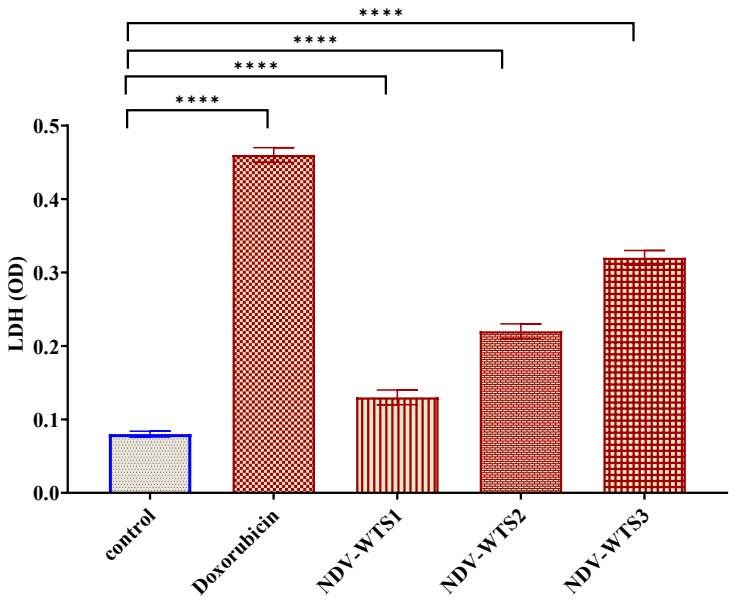


Figure 2. LDH release from TC-1 cells under the influence of various treatments. This figure provides a comprehensive overview of how different therapeutic interventions affect the integrity of TC-1 cells, as indicated by the levels of LDH released into the surrounding medium. Data are plotted as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

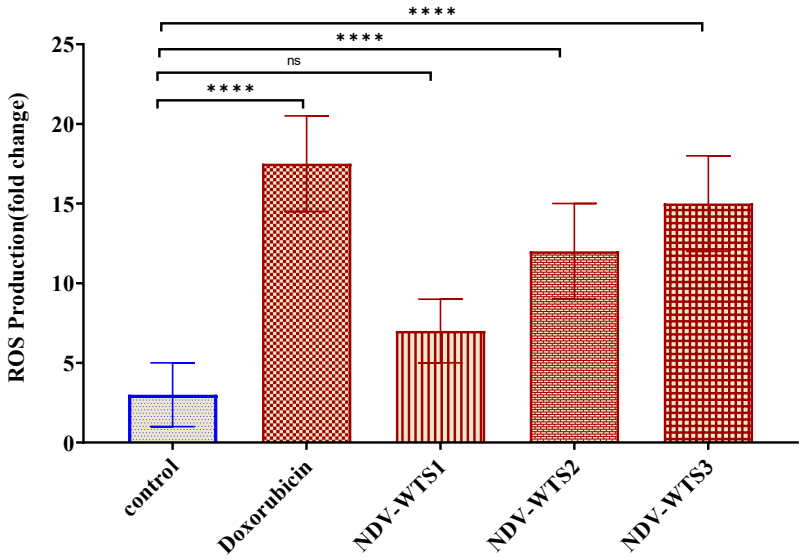


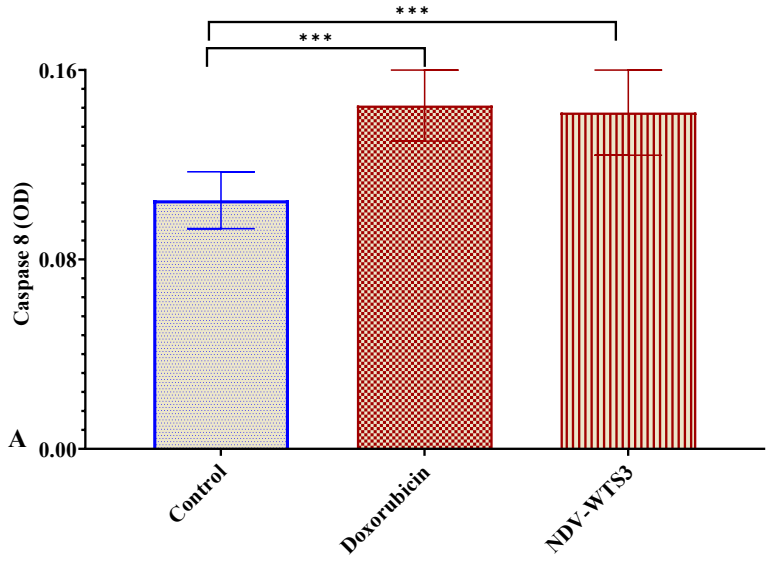
Figure 3. Production of ROS from TC-1 cells under the influence of various treatments. This figure provides a detailed examination of how different therapeutic interventions impact oxidative stress levels within the cells. Data are plotted as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Caspase Activity

Caspases are crucial cysteine proteases that regulate apoptosis and inflammation. They play a vital role in cellular processes, mediating programmed cell death and influencing various pathological conditions, including cancer. The results indicate a noteworthy increase in the levels of Caspase 8 and 9 in TC-1 cells that were treated with NDV-WTS, as illustrated in Figure 4A and 4B (p<0.0001). This significant elevation suggests that the treatment with NDV-WTS effectively activates the intrinsic and extrinsic apoptotic pathways, which are primarily mediated by Caspase 8 and 9.

Real-time PCR

The highest concentration of NDV-WTS (MOI 4) increased the expression of *Bax*, *Casp8*, and *Casp9* by 3.76-, 5.41-, and 1.76-fold change (p<0.05), respectively. The relative gene expression levels of *Bcl2* were decreased by 1.231-fold change in the NDV-WTS-treated TC-1 cells compared to the control (p<0.05) (Figure 5A–D).



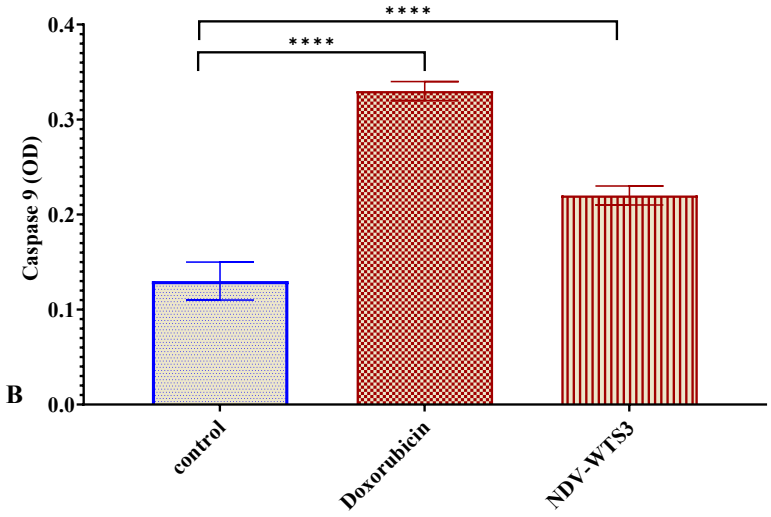
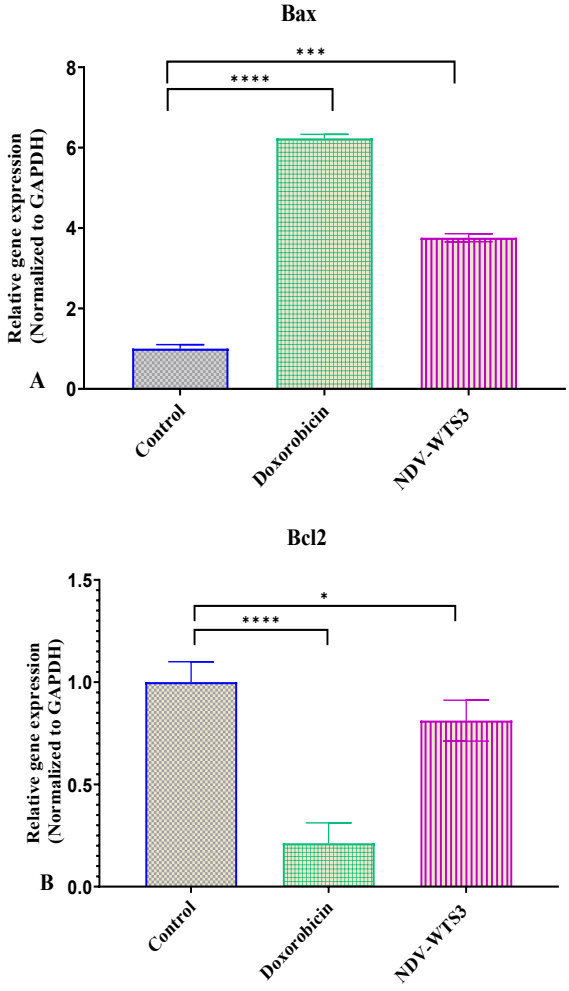


Figure 4. Activity levels of Caspase 8 and Caspase 9 in TC-1 cells. A. Caspase 8 activity. B. Caspase 9 activity. The figures provide a comprehensive overview of the activity levels in TC-1 cells subjected to different treatments. Data are plotted as mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



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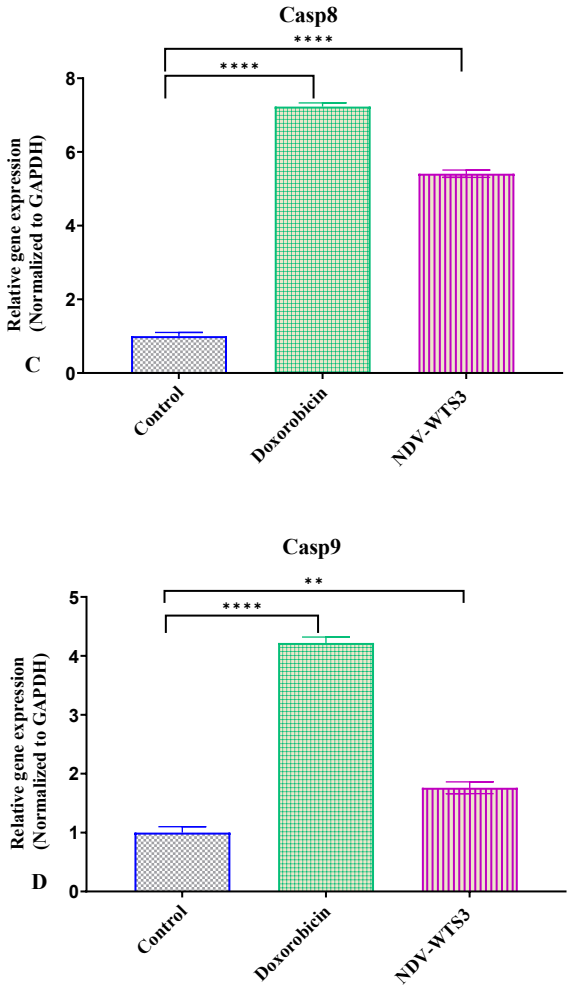

Figure 5. Relative expression of *Bax*, *Bcl2*, *Casp8*, and *Casp9* in NDV-WTS-treated TC-1 cells vs control TC-1 cells, normalized to *Gapdh*. Expression of *Bax*, *Casp8*, and *Casp9* were significantly upregulated in NDV-WTS-treated TC-1 cells vs control, and expression of *Bcl2* was significantly downregulated in NDV-WTS-treated TC-1 cells vs control.

DISCUSSION

OVs represent a promising strategy in cancer therapy, as they selectively infect cancer cells while sparing healthy tissues, stimulating strong antitumor immunity. Several OVs have demonstrated encouraging results in both in preclinical and early clinical trials. Notably, NDV stands out as a leading oncolytic agent due to its good safety profile and its ability to induce cell death that activates both local and systemic anticancer immune responses. NDV is an avian virus that the mammalian immune system cannot eliminate due to existing antibodies in the blood. This gives NDV an advantage over other commonly used virotherapy viruses, such as adenoviruses and measles, which face limitations from the body's natural immune response. NDV's natural tumor tropism means it does not require additional viral delivery techniques and can effectively evade the host's antiviral immune response.[13] NDV triggers apoptosis in cancer cells and activates the innate immune system by increasing cytokine production and enhancing antigen presentation. Its hemagglutinin-neuraminidase protein boosts cytolytic T cell responses against tumor cells. NDV transforms the immunosuppressive tumor microenvironment into a proinflammatory one, thereby, promoting antitumor responses. Even low levels can inhibit tumor growth. Additionally, NDV allows for the insertion of foreign genes without removing its own viral genes. Human seropositivity to NDV is low compared to other

oncolytic viruses. Research indicates that NDV can induce various forms of cell death in cancer cells, including apoptosis, autophagy, and necrosis/necroptosis. This highlights the virus's ability to effectively target and eliminate malignant cells through multiple mechanisms.[14,15] The current study aimed to evaluate the effects of wild-type NDV on LDH secretion and apoptosis induction in TC-1 cells. In this experimental study, TC-1 cell lines were cultured under laboratory conditions. The findings revealed that wild-type NDV significantly reduced cell viability (p<0.05) while prompting apoptosis, increasing ROS levels, LDH release, and Caspase 8 and 9 activities compared to the control group. Additionally, molecular analyses indicated that treatment with NDV significantly elevated the levels of *Bax*, *Casp8*, and *Casp9*, while significantly decreasing *Bcl2* expression compared to the control group. Cuadrado-Castano et al, 2015 revealed that the NDV Lasota strain exhibits cytotoxic effects on MCF-7 tumor cells and increases apoptosis by the intrinsic pathway.[16] Yurchenko et al, 2018 showed that NDV significantly decreases the proliferation index and significantly increases the apoptosis of the A549 cell line by enhancement of NDV replication in human non-small-cell lung cancer.[17] Determining cell viability is essential aspect of cellular models studies, with assays focusing on membrane integrity being the most commonly employed methods.. Although loss of plasma membrane integrity is considered irreversible, it does not always indicate cell death, as other biochemical changes may occur without leading to cell death. Membrane integrity can be assessed by measuring the activity of cytosolic enzymes such as LDH, which are released into the culture supernatants following membrane disruption. Under physiological conditions, intracellular ROS levels are tightly regulated to prevent cellular damage. However, elevated ROS levels can lead to tumor cell death by causing nonspecific damage and by specifically triggering apoptosis through death signaling pathways.[18] ROS are mainly generated in mitochondria and play a vital role in cell death and oxidative stress responses. Elevated ROS levels can enhance apoptosis and are also associated to autophagic cell death, characterized by the formation of autophagosome. Recent studies have demonstrated that ROS secretion can induce apoptosis and mitochondrial dysfunction in gastric adenocarcinoma cells infected with oncolytic adenovirus.[19] Apoptosis is triggered when cytotoxic agents activate death signaling pathways, leading to mitochondrial dysfunction and subsequent caspase activation. Caspase activation is essential for the induction of apoptosis by anticancer agents. Caspases are crucial in both the initiation and execution phases of in various cell types.[20] It has been shown that the NDV toxicity is mediated by oxidative stress in different cell lines. Furthermore, several studies have reported that oncolytic NDV can stimulate both the intrinsic and extrinsic apoptosis pathways. However, some studies have also reported different results. For instance, Keshavarz et al, 2020 reported that NDV can induce apoptosis via the mitochondrial intrinsic pathway.[21] Kumar et al (2012) reported that NDV induces apoptosis in HeLa cells by extrinsic TRAIL receptor pathways and activation of the caspase cascade.[22] The observed caspase levels in the doxorubicin-treated group are consistent with the findings of Sharifi et al (2015), who revealed that the main mechanism by which doxorubicin induces apoptosis is via the mitochondrial pathway. mitochondrial pathway.[23] In conclusion, the findings demonstrate that NDV-WTS exert a significant impact on cell viability, leading to increased apoptosis and elevated levels of ROS. The observed rise in LDH release and Caspase 8 and 9 activities, further supports the apoptotic effects of NDV and suggests that NDV may primarily activate the intrinsic and extrinsic apoptotic pathways. Molecular analyses showed NDV treatment upregulates apoptotic factors such as *Bax*, *Casp8*, and *Casp9* while decreasing *Bcl2*. Collectively, these results highlight the therapeutic potential of NDV-WTS in cancer treatment.

STATEMENT OF ETHICS

Baqiyatallah University of Medical Sciences ethical committee confirmed this study's protocols (Ethics: IR.BMSU.REC.1397.354).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

Upon reasonable request can be submitted via email at: h.smaili69@yahoo.com.

AI ASSISTANCE DISCLOSURE

Not applicable

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