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Apoptotic activity of Newcastle disease virus in comparison with nisin A in MDA-MB-231 cell line

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Abstract

Given the development of drug-resistant cancer cells, designing alternative approaches for cancer treatment seems essential. In this study, we evaluated the anti-tumor effects of nisin A and Newcastle disease virus (NDV) on triple-negative MDA-MB-231 cell line. The MDA-MB-231 cell line was separately and in combination subjected to the different concentrations of a Vero-adapted NDV (JF820294.1) and nisin A. The oncolytic effects of these treatments were analyzed by different cytotoxic and apoptosis techniques including trypan blue staining, MTT assay, acridine orange (EB/AO) staining, colony assay and flow cytometry over time. Nisin A at doses of more than 20.00 μg mL^{-1} could represent the anti-viral effects and interfere with the oncolytic activity of NDV. Moreover, the analyses indicated that the anti-proliferative and cytotoxic features of combination therapy were stronger than those of individual NDV groups. However, the most apoptotic effect was seen in NDV experimental groups. Taken together, the results from cytotoxicity tests, flow cytometry and colony assay showed that either of the oncolytic agents had significant effects at low concentrations 72 hr post-treatment. Thereby, they had the potential to be used as new approaches in cancer treatment.

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Introduction

Breast cancer is a major cause of women's death and an important health problem, worldwide. More than 450,000 deaths as a result of breast cancer are recorded in both developed and developing countries.[1] The aggressive, complex and resistant nature of the breast tumor cells has made many challenges in the treatment protocols. Surgery, chemotherapy and radiation therapy are three conventional approaches for cancer treatment. However, they are invasive and not tumor-specific. Further, these strategies have toxic effects and are deleterious for normal cells.[2] Anti-estrogens, aromatase inhibitors, and anti-angiogenesis drugs as the potential choices for the treatment of breast cancers, required long-term administration and may cause serious side effects such as cardiotoxicity. Moreover, these agents are expensive and not applicable in patients with advanced cancers like triple-negative MDA-MB-231 cell line. Lack of estrogen and progesterone receptors and the inability to produce enough HER2 protein make MDA-MB-231 cells resistant to drug treatment. In this line, exploring the safe novel agents with the ability to overcome multidrug-resistant tumor cells will be an enormous achievement.[3-6] In this context, Newcastle disease virus (NDV) and nisin as two oncolytic agents have attracted interests from researchers in recent years.

The NDV is an enveloped, negative-sense single-strand RNA virus that belongs to the *Paramyxoviridae* family and *Orthoavulavirus* genus. The NDV genome codes six major proteins including large polymerase protein (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P) and nucleocapsid protein (NP), respectively (5′ → 3′ direction). Based on the virulence NDV isolates are classified into lentogenic, mesogenic and velogenic pathotypes.[7,8] Despite the severe disease in avian species caused by NDV, it is known to have strong oncolytic effects against the mammalian cancerous cell. Selective anti-cancer characteristics and safety for human use have made NDV a suitable choice for oncolytic virotherapy.[9] The oncolytic ability of NDV was first identified in the 1950s. Prince and Ginsberg proved that NDV has preferential apoptosis

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effects on Ehrich ascites carcinoma cells.[10] Fifteen years later in 1967, Eaton *et al.* confirmed this idea by testing NDV on lymphoma and leukemia murine models.[11] NDV can replicate selectively and induce apoptosis with defective IFN-γ signaling pathways.[12] Indeed, to enhance the effectiveness of NDV viral therapy genetically modified viruses were generated over the last few years.[13,14]

On the other hand, nisin is a prokaryotic origin peptide that is produced by Gram-positive bacteria such as *Lactococcus* species.[15,16] Its safety for human use has been approved by Food and Agriculture Organization (FAO) and World Health Organization (WHO) since 50 years ago.[17] Also, Food and Drug Administration (FDA) has recognized it as a safe molecule for clinical use.[18] Nisin is a promising antimicrobial peptide (AMPs) with approved anti-tumor effects.[19-23] Also, nisin has immunomodulatory functions and can activate the host adaptive immune system.[24] These features along with the broad-spectrum activity have made nisin A considerable novel agent for cancer treatment and some antibiotic-resistant diseases.[25,26] Moreover, the synergic effect of different natural and modified nisin variants has been assessed, so far. Evaluation of the synergic effect of doxorubicin and nisin combination against 7,12-dimethylbenz(a)anthracene (DMBA)-induced skin carcinogenesis,[22] performing Argentum-nisin nanoparticle[27] for activation of macrophage cells and analysis of cytotoxic effects of nisin-loaded poly lactic acid-poly ethylen glycol-poly lactic acid (PLA-PEG-PLA) nanoparticles on gastrointestinal, hepatic and blood cancer cell lines,[28] are examples of the recent studies.

Durable responses and the oncolytic effect of treatment on distant lesions require stronger activation immune responses which can be achieved by the combination of viral oncolytic therapy and other forms of treatments including cytotoxic chemotherapy, radiation therapy and adoptive T cell therapy.[29-39] In addition to improved therapeutic responses, the toxicity-dose limitation can significantly be decreased through combination therapy which has been confirmed via preclinical studies.[40]

Here, we evaluated and compared the oncolytic effects of nisin A and NDV separately and in combination on the triple-negative MDA-MB-231 breast cancer cell line. This study was aimed to advance our understanding of the apoptosis and cytotoxicity effects of these agents on the MDA-MB-231 cell line. Moreover, this research addressed the synergistic and inhibitory interactions between NDV and nisin A in combination therapy.

Materials and Methods

Cell culture. The human breast cancer (MDA-MB-231) cell line was cultured (Pasteur Institute, Tehran, Iran) in RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) with 10.00% fetal bovine serum (FBS; Sigma-Aldrich), 10.00 U mL^{-1} of cell culture penicillin-streptomycin (Sigma- Aldrich) and 1.00 µg mL^{-1} amphotericin (Sigma-Aldrich) and incubated under an atmosphere of 5.00% CO_2 and 95.00% air at 37.00 ˚C.

NDV propagation. The NDV used in this experiment was isolated from Fars province poultry fields (JF820294.1)[41] adapted to the Vero cell line. For the expansion of the virus, first, the Vero cells were plated in a T75 culture flask. When cells reached 70.00% confluency, supernatant medium (DMEM; Sigma-Aldrich) was removed and 200 µL NDV (passage 4) was added to the flask. After one hour incubation, 15.00 mL DMEM medium with 10.00% FBS, 10.00 U mL^{-1} of cell culture penicillin-streptomycin (CBSAlife, Winnipeg, Canada) and 1.00 µg mL^{-1} amphotericin (Sigma-Aldrich) were poured into the flask and incubated for five days under an atmosphere of 5.00% CO_2 and 95.00% air at 37.00 ˚C. Then, the cell suspension was frozen and thawed three times to break up the cells. Ultimately, the cell lysate was centrifuged and the supernatant containing Passage 5 NDV was collected.

Experimental design. MDA-MB-231 cell line was cultured in RPMI medium. After 24 hr of incubation, the culture media were replaced with three different concentrations of ultra-pure nisin A (20.00, 40.00, and 80.00 µg mL^{-1}; Handary SA., Brussels, Belgium) was prepared. The cells were also treated with NDV at the multiplicity of infection (MOI) of 0.22, 0.44, and 0.88 separately. In the next step, NDV at MOI 0.22 was employed in combination with 20.00, 40.00, and 80.00 µg mL^{-1} of nisin A and the probable synergetic effects of them were compared. The results of all the treatments were surveyed after 24, 48, and 72 hr. The oncolytic effects were measured with colony assay, ethidium bromide/Acridine orange (EB/AO) method, flow cytometry and cytotoxicity tests including MTT and trypan blue staining.

Trypan blue staining. The cells were seeded in 6-well plates with a density of 10^5 cells per well and incubated for 24 hr. Subsequently, the treatments were done for each well and the results were recorded as follows: First, the cells were detached from the culture plates using PBS containing 1.00 mM EDTA (Sigma-Aldrich) and centrifuged (Eppendorf 5417R, Hamburg, Germany) at 200 *g* for 10 min and the obtained pellets were re-suspended in PBS. In the end, 100 µL cells suspension of each treatment was mixed with 300 µL of prepared trypan blue dye (Merck KGaA, Darmstad, Germany) and microscopic changes were observed using light microscopy which represented live and dead cells with clear and dark colors, respectively.

MTT assay. For MTT (3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, MDA-MB-231 cells were seeded in 96-well plates at a density of 5.00×10^3 cells per well. After 24 hr, the cells were exposed to various treatments as described earlier. After that, the supernatant medium was removed and the reaction was continued in presence of fresh phenol red free Roswell Park Memorial

Institute (RPMI) medium and 12.00 mM MTT reagent (Sigma-Aldrich). After 4 hr of incubation, the MTT containing culture medium was replaced with 50.00 µL dimethyl sulfoxide (DMSO; Sigma-Aldrich) and the plates were incubated for further 10 min. Finally, the absorbance of each well was recorded by ELISA reader (BDSL Immunoskan PLUS Labsystems, Vantaa, Finland) at 570 nm and the viability of the cells (%) was calculated as follows:

Cell viability = (OD of treated sample / OD of control) × 100

where, control is an untreated sample and OD is the optical density.

Ethidium bromide/acridine orange (EB/AO) staining. According to Ribble *et al*. protocol,[42] the percentage of live, apoptotic and necrotic cells was determined by EB/AO staining. The cells were seeded in 6-well plates with a density of 10^5 cells per well. After 24 hr of incubation, the cells were subjected to various treatments as described earlier. Subsequently, 24, 48, and 72 hr post-treatment, the cell suspensions were prepared as described in current protocols of immunology.[43] Next, 25.00 µL of the suspension was stained with 25.00 µL of EB/AO. Finally, the stained cells were assessed and counted with a fluorescence microscope.

Colony assay. Colony assay is a cell biology technique studying the antiproliferative effects of different agents on cells. Here, we assessed the proliferation and viability rate of the MDA-MB-231 cell line after the mentioned treatments by colony assay. For this purpose, the cells were seeded on 6-well plates at a cell density of 2.00×10^3 per well. After 24 hr of incubation, the cells were exposed to the treatments. Then the cultures were incubated for the other 10 days. After incubation time, colonies were recognized with 15.00 mM crystal violet (Sigma-Aldrich) and the ratio of the colonies containing less than 50 cells to all formed colonies was calculated.

Flow cytometry. Flow cytometry was conducted to evaluate the apoptosis and necrosis rate following various treatments. Briefly, MDA-MB-231 cell line was cultured in 12.50 cm^2 cell culture flasks and treated as described above. Then the cells were harvested and washed with cell staining buffer of BioLegend (San Diego, USA). Afterward, 0.25×10^7 cells mL^{-1} were resuspended in Annexin V binding buffer (BioLegend, San Diego, USA) and transferred to the 5.00 mL tubes. After that, FITC Annexin V was added to the cell suspension and mixed gently for 15 min. Finally, the Binding buffer was also added to the prepared tubes and analyzed by BD FACS Calibur flow cytometer (MACSQuant10; Miltenyi Biotec, Teterow, Germany).

Statistical analysis. In this study, all experiments were performed three times. Statistical analysis was done with SPSS Software (version 16.00; SPSS, Inc., Chicago, USA). Results of different groups were evaluated by One-way ANOVA and Tukey tests. A simple repeated measurement test was used for comparing the results obtained by the time at each treatment group. Data were reported as mean ± SE and a $p < 0.05$ was considered significant.

Results

Trypan blue staining. Viability rates of treated and untreated control MDA-MB-231 cells were compared to each other by bright field light microscopy using trypan blue staining. All the treatments, except for nisin 20, showed statistically significant differences ($p < 0.05$) with the control groups after 24, 48, and 72 hr post-treatment. Deadly effects of nisin for all three concentrations were increased remarkably over time to the extent that 100% cell destruction was observed under nisin 80 treatment after 48 and 72 hr. Cytotoxic effects of combination therapy with NDV 0.22 + nisin 40 and NDV 0.22 + nisin 80 were enhanced compared to NDV at MOI of 0.22. This difference was not significant about NDV 0.22 + nisin 20 and NDV 0.22 experimental groups (Fig. 1).

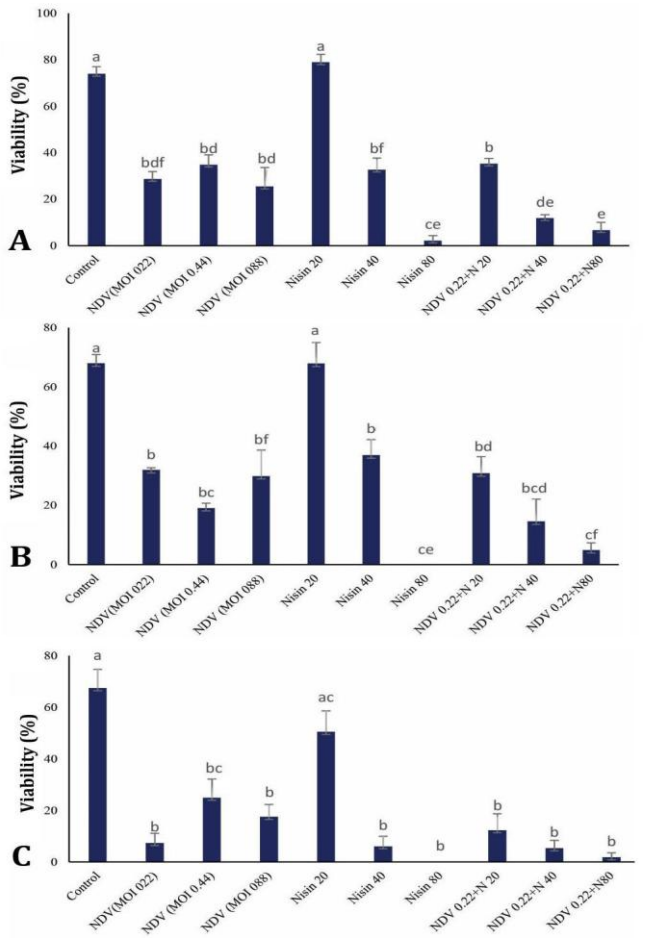

Fig. 1. Cytotoxicity of different treatments on MDA-MB-231 cells using trypan blue staining. **A**, **B,** and **C)** represent data obtained after 24, 48, and 72 hr, respectively. Data are expressed as mean ± SE and all the treatments are performed in triplicate. [a-f] Different letters indicate significant differences ($p < 0.05$).

MTT assay. The effect of time on cellular degradation was more pronounced via MTT assay and as can be seen in Figure 2. The differences among and within the treatment groups were more significant after 72 hr. This result was clearer about the combination experimental groups that were completely distinct from each other after 72 hr. Indeed, more anti-proliferative effects were observed applying combination treatment of NDV 0.22 with nisin 40 and 80 after 48 hr which remained remarkable for NDV 0.22 + nisin80 after 72 hr.

EB/AO staining. All NDV groups caused significant apoptosis rates compared to the control group after 48 hr post-treatment. Moreover, the percentage of viable cells was decreased remarkably after 48 hr in nisin 20 and after 24 hr in nisin 40 and nisin 80 experimental groups (Fig. 3). The same results were obtained applying combination treatments with NDV 0.22 and different concentrations of nisin. Furthermore, the apoptosis was significantly higher in the NDV 0.22 + nisin 40 and NDV 0.22 + nisin 80 compared to the NDV 0.22 + nisin 20 experimental group.

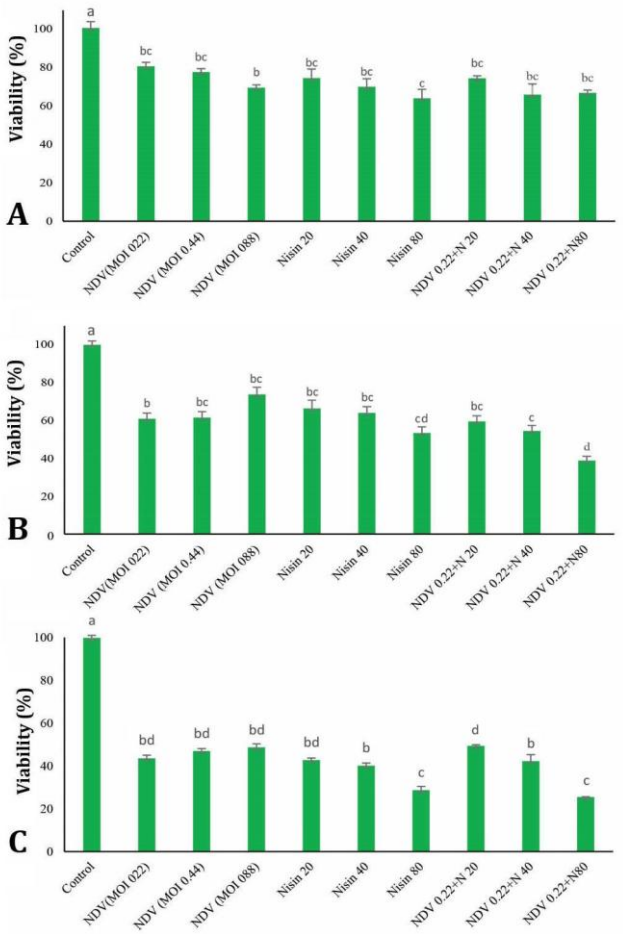

Fig. 2. Cytotoxicity of different treatments on MDA-MB-231 cells using MTT assay. **A**, **B,** and **C)** represent data obtained after 24, 48 and 72 hr, respectively. Data are expressed as mean ± SE and all the treatments are performed in triplicate. [abcd] Different letters indicate significant differences ($p < 0.05$).

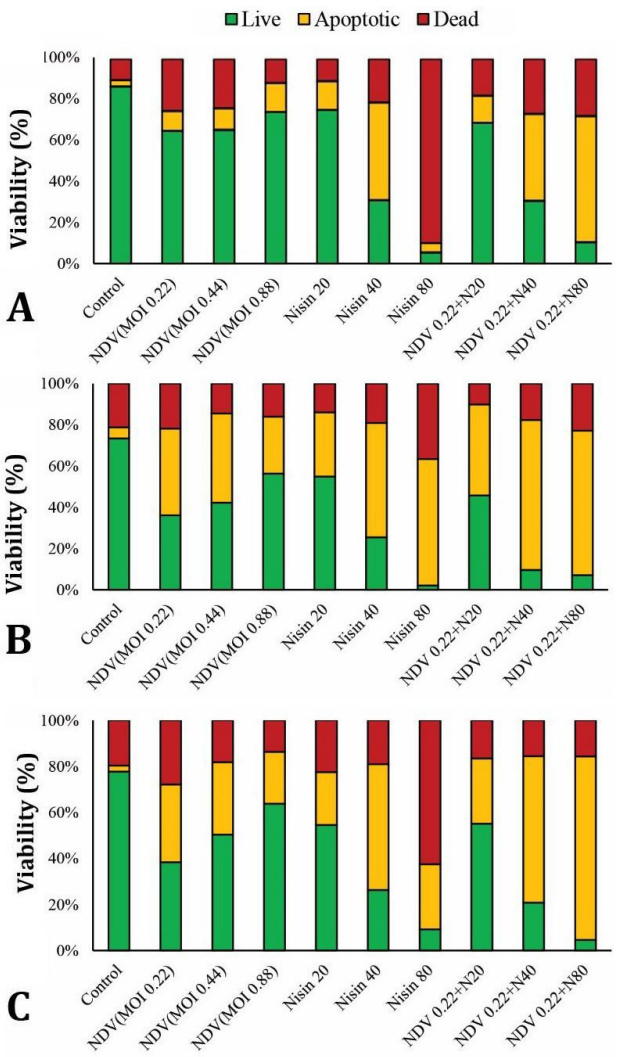

Fig. 3. Cytotoxicity and apoptosis of different treatments on MDA-MB-231 cells using EB/AO method. **A**, **B,** and **C)** represent data obtained after 24, 48 and 72 hr, respectively. Data are expressed as mean ± SE and all the treatments are performed in triplicate.

Colony assay. The results of the colony assay were compatible with the previous cytotoxicity tests. In this experiment, colonies with less than 50 cells were counted for each treatment. All treatments showed a significant difference from the control group. All of the counted colonies for Nisin 80 and NDV 0.88 included less than 50 cells. However, both treatments showed no statistically significant difference with 40.00 µg mL^{-1} nisin and MOI 0.44 of NDV. The anti-proliferative effects of all three combination groups were strongly more and remarkably different in comparison with NDV 0.22 (Fig. 4).

Flow cytometry. Annexin-V FITC assay was performed for evaluation of MDA-MB-231 cells viability condition in four levels of live, early apoptosis, late apoptosis and necrosis treated with 10 different groups as mentioned before. Overall, the rate of apoptosis was more

than necrosis for each group and this difference was increased considerably over time like the other three tests. The cell necrosis was unremarkable compared to the control untreated groups. The ratio of apoptotic-to-necrotic cells for all three NDV groups was more than other treatments. Surprisingly, the percentage of viable cells in the combination with treatment groups was more than this value at NDV 0.22 group and the number of viable cells at these NDV groups was the lowest. More precisely, the number of viable cells at combination groups of NDV 0.22+nisin 20 and NDV 0.22+nisin 40 was notably different compared to NDV 0.22. These differences extended and generalized to all three combined groups at 48 and 72 hr post-treatment (Fig. 5). Although early apoptosis for nisin 40 and nisin 80 had an increasing rate over time, this process for NDV groups was faster than other groups. Combination treatments substantially decreased the amount of early apoptosis compared to NDV with MOI 0.22 so that early apoptosis at the combination group of NDV 0.22 + nisin 20 was significantly less than NDV 0.22 group in all three times of treatment. Moreover, the rate of early apoptosis was lower than the MOI of 0.22 NDV 48 hr post-treatment. Furthermore, late apoptosis at NDV groups was considerably more than that at nisin 40 and nisin 80 after 72 hr. The rate of late apoptosis was increased in parallel with time (Fig. 5).

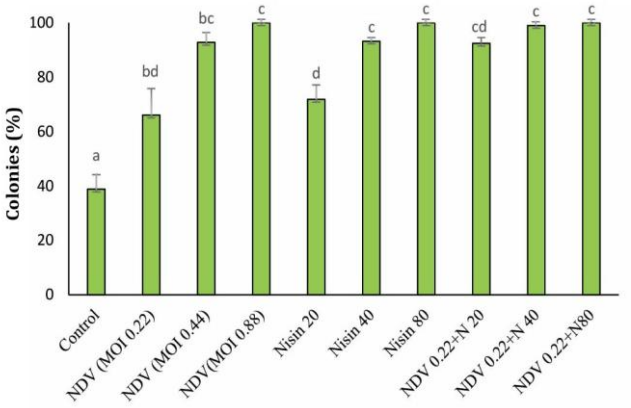

Fig. 4. Effect of different treatments on colony formation of MDA-MB-231 cells after 24 hr. Colony values (%) with less than 50 cells are presented on the vertical axis. Data are expressed as mean ± SE and all the treatments are performed in triplicate. [abcd] Different lower cases indicate significant differences ($p < 0.05$).

Discussion

In this study, the effects of different concentrations of nisin, NDV and combinations of them on MDA-MB-231 cell destruction were studied over time by different detection methods. Different approaches help us to evaluate the results more precisely.

Some oncolytic viruses (OVs) such as NDV, Maraba virus, B18R-deficient vaccinia virus, and recombinant

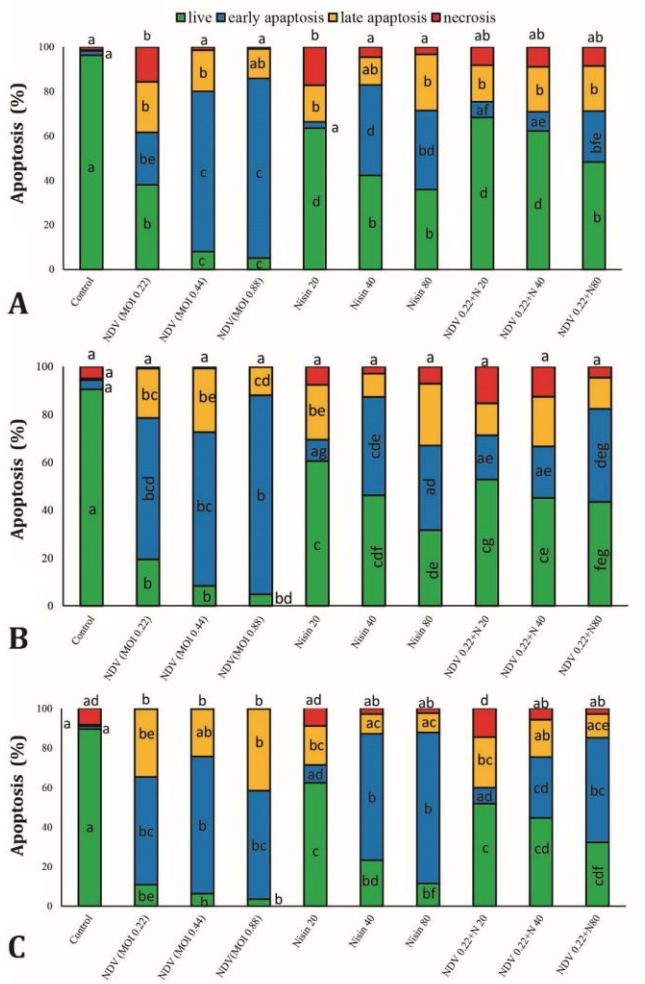

Fig. 5. Cytotoxicity and apoptosis of different treatments on MDA-MB-231 cells using flow cytometry. **A**, **B,** and **C)** represent data obtained after 24, 48 and 72 hr, respectively. Data are expressed as mean ± SE and all the treatments are performed in triplicate. [a-g] Different lower cases indicate significant differences ($p < 0.05$).

vesicular stomatitis virus (VSV-ASMEL) have been used in combination with therapeutic antibodies to improve the anti-cancer treatment efficacy of immune checkpoint blockade. These viruses trigger immune responses by recruiting tumor-infiltrating lymphocytes (TILs) and inducing the release of pro-inflammatory cytokines, soluble tumor antigens and danger signals.[30,31,33] In a preclinical study, Zamarin *et al.*[39] proposed that combination therapy of B16 melanoma with localized oncolytic NDV and CTLA-4 blockade stimulated the tumor-specific lymphocytic infiltration into both the injected tumor and distant tumors and consequently increased the sensitivity of tumor cells to systemic therapy with CTLA4 blockade. As the only FDA-approved OV, T-VEC is used for the treatment of advanced melanoma. This recombinant herpesvirus was used in a phase Ib clinical trial in the combination with anti-CTLA4 antibody ipilimumab.

Interestingly, the patients showed enhanced and durable responses to this treatment compared to administration ipilimumab alone. Of note, no dose-limiting toxicities and increased adverse effects were observed.[36] Indeed, the combination treatment of various solid tumors with coxsackievirus (CVA21) and ipilimumab/ pembrolizumab presented the same promising responses, as well.[38] Other than immune checkpoint blockade, concomitant viral therapy with chemotropic agents such as cisplatin and 5-fluorouracil have shown that it can enhance tumor cell death.[32,37] Furthermore, in a study by Adusumilli *et al*. the combination of HSV-1 and radiation therapy increased antitumor activity in mice. The synergistic effect is due to cell DNA damage caused by radiation. In turn, OV impedes DNA repair mechanisms by suppression of DNA damage response proteins.[29,35]

Pre-treatment of tumors with OVs help to prepare the anti-cancer immune microenvironment for adoptive cellular therapy (ACT). The OVs enhance T cell recruitment and induce necrosis and pyroptosis of tumor cells resulting in the spreading of tumor neoantigens and priming of neoantigen-specific $CD8^+$ T cells. These viruses also up-regulate the expression of MHC class I and consequently promote the function and survival of T cells during cancer treatment.[34]

In general, nisin at the lowest concentration, 20.00 μg mL^{-1}, had small deleterious effects on the cell lines compared to the control and other treatment groups. It could be explained by the maximum tolerable concentration (MTC) of nisin which is supposed to be 25.00 μg mL^{-1}.[44] However, two other treatment groups with concentrations of 40.00 and 80.00 μg mL^{-1} of nisin had remarkable destructive effects on the cells. The efficiency of nisin at doses 40.00 and 80.00 μg m mL^{-1} was observed after 72 hr and 24 hr, respectively. These findings, along with the results of another study,[44] showed that the effect of nisin did not diminish at least during 72 hr. Therefore, the use of nisin at the minimum effective concentration of 40.00 μg mL^{-1} but over a longer period, can have an efficient oncolytic impact and prevent possible side effects on normal cells caused by higher concentrations of nisin.

The same results were obtained for the NDV treatment groups and all of the experiments demonstrated the importance of the time for the effective oncolytic function of the virus as well as nisin. This finding was clearer by the results of the flow cytometry assay. It seemed that NDV at MOI 0.22 had an acceptable function for the cell line killing and if it was used for an appropriate period (at least 72 hr post-infection), it would be a good choice for inducing MDA-MB-231 tumor cells death. However, the high cytotoxicity effects of NDV 0.22 were represented after 48 hr according to EB/AO staining.

The role of the time was more pronounced for MTT assay than trypan blue staining. This is due to the differences in the mechanism of action of these two cytotoxic assays which are based on metabolic activity and integrity of cells, respectively.[45] If the integrity of cells is lost at early stages, trypan blue dye will exclude through the membrane and non-viable cells exhibit a blue cytoplasm. This may happen while the enzymatic activity of the cells has not been lost, yet. Therefore, decreasing cell viability will be more distinguishable at MTT assay over time compared to trypan blue staining.

Decreasing of cell viability at NDV 0.22 + nisin 20 group was also observed compared to nisin 20. It was self-evident that a combination of NDV (MOI 0.22) with nisin 40 and nisin 80 had more desirable effectiveness compared to combination therapy with nisin 20. Furthermore, time had an important role in the interpretation of the following results. These results were more reliable 72 hr post-treatment. Apoptosis had the main role in MDA-MB-231 cell line killing caused by NDV or nisin. However, regarding increment in the number of viable cells and decrement in the amount of early apoptosis at combination experimental groups, it seemed that although these treatments had more cytotoxicity and the anti-proliferative activity, the apoptosis rate at NDV of MOI 0.22 group was enhanced significantly.

The concentration of nisin may change the oncolytic process of the treatment. Flow cytometry results obtained from combination therapy indicated that the major cause of cell apoptosis at low doses of nisin (20.00 μg mL^{-1} in this study) was enhanced because of the NDV function. Conversely, in high concentrations, the negative effect of nisin on the virus prohibited the synergic effects of these two anti-cancer agents. This idea was supported by the results of two other combination treatment groups of NDV + nisin40 and NDV + nisin80. In these treatment groups, the percentage of viable cells was even more than that at nisin (40.00 and 80.00 μg mL^{-1}) and NDV groups. Overall, it could be inferred that nisin had cytotoxic and anti-viral effects at doses of more than 25.00 μg mL^{-1}. This deduction was in agreement with another study published by Małaczewska *et al.* [44] Nevertheless, results recovered from EB/AO staining showed that oncolytic effects of NDV 0.22 + nisin 40 and NDV 0.22 + nisin 80 were more than NDV 0.22 experimental groups. This might be due to the difference in the preparation process of the sample during flow cytometry assay and EB/AO staining.

The finding of colony assay as an extensively used technique[46] was consistent with the trypan blue, MTT and EB/AO staining results exhibiting that combination treatment groups had greater anti-proliferative effects on MDA-MB-231 cell line in comparison with NDV treatments.

The safety of nisin as a food preservative regarding eukaryotic cells has been approved by health organizations.[26] However, some new studies have shown that it can modulate human and other eukaryotic immune systems.[24] On the other hand, the results of this study

showed that the nisin induced apoptosis in MDA-MB-231 cell line. Therefore, further investigations about the exact mechanism of nisin on eukaryotic cells and its possible anti-viral effects, were seemed essential. Some studies suggested that overexpressing of different receptors on the different cell lines could change the functions of oncolytic agents.[27,28] Thereby, the effectiveness of nisin and NDV on a wide range of cell lines and their possible negative effects on normal cells should be more evaluated. Moreover, the Synthesis of targeted NDV and targeted nisin may be an interesting topic for future research.

In conclusion, Nisin at amounts of more than 20.00 μg mL^{-1} (40.00 μg mL^{-1} in this study) had significant oncolytic effects on MDA-MB-231 cell line after 72 hr. The same results were obtained for NDV with MOI 0.22. In addition, nisin A at doses of more than 20.00 μg mL^{-1} had an anti-viral effect on NDV. Despite the anti-proliferative power of combination therapies, it seemed that the apoptotic effects of NDVs, which had the main role in the MDA-MB-231 cell death process, were more than other treatments. Thereby, combination treatment of MDA-MB-231 cells with both NDV and nisin could decrease the necessary doses for treatment and possible side effects of these two oncolytic agents on normal cells.

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Conflict of interest

The authors declare no conflict of interest.

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