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ORIGINAL ARTICLE

Synergistic effects of *Bacillus coagulans* and *Newcastle disease virus* on human colorectal adenocarcinoma cell proliferation

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ABSTRACT

Background and Objectives: Colorectal cancer (CRC) is a common type of cancer that has a high death rate and is becoming more common in developed countries. Currently, there are several treatment options available for CRC patients, and clinical trials are being conducted to improve conventional therapies. This study investigates the combined impact of *Bacillus coagulans* (B.C) and *Newcastle disease virus* (NDV) on the growth of human colorectal adenocarcinoma cells (HT29 cell line).

Materials and Methods: The HT29 cell line was cultured under controlled laboratory conditions. They were treated with Fluorouracil (5-FU), NDV, and B.C., after which various assessments were conducted to determine the effects of these treatments. These assessments included MTT assay for cytotoxicity, evaluation of cell viability, and measurement of caspase 8 and 9 activity levels. The significance of the data was determined at a threshold of $P<0.05$ following analysis.

Results: The usage of NDV and B.C significantly increased cell death and reduced cell growth in the HT29 cell line, when compared to the control group. Moreover, the combined application of NDV and B.C along with 5-FU exhibited a synergistic effect in decreasing the proliferation of HT29 cells. Additionally, the results indicated that intrinsic apoptosis pathway was activated by B.C and NDV.

Conclusion: It appears that utilizing oncolytic viruses (OV) and bacteria in conjunction with chemotherapy drugs could potentially aid in reducing the growth of colorectal cancer cells. However, further research is necessary, including animal studies, to confirm the efficacy of this treatment method.

Keywords: Colorectal cancer; HT29 cell line; Oncolytic virus; *Newcastle disease virus*; *Bacillus coagulans*; Apoptosis; Caspase; Cytotoxicity

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INTRODUCTION

Colorectal cancer (CRC) is a common and deadly cancer that causes many fatalities each year. Surgical procedures and chemotherapy are the main treatments, but the prognosis is often poor if cancer has spread. Surgery is the first step in treating colon cancer (CC), followed by chemotherapy and radiation therapy (1, 2). 5-FU treatment improves survival in various cancers, especially CRC. Its active metabolites disrupt DNA and RNA synthesis through the folate metabolic pathway (3). CRC symptoms include changes in bowel patterns, blood in stool, abdominal discomfort, fatigue, and weight loss (4). Drug resistance is a common problem in CC patients, reducing the efficacy of anticancer drugs. Targeted therapy is a promising approach to improve survival rates. Additionally, using a drug delivery system with complementary agents from plants, viruses, and bacteria may reduce complications (5). Probiotics and Oncolytic viruses (OVs) are novel cancer treatment paradigms. OVs selectively replicate in tumor cells, impeding tumor progression (6). Talimogene laherparepvec (Imlygic® or T-VEC) is an OV designed for melanoma treatment. It is administered via direct tumor injection, increasing immune cell production and reducing herpes transmission risk (7). NDV is an avian paramyxovirus types I virus belonging to the genus Avulavirus, with potential as an oncolytic agent (8). It can combat various tumor types, including lymphoma, glioblastoma, and liver cancer, and can even target tumor stem cells and dormant tumor cells (9). Its impact on cell growth is distinct from its RNA transcription and translation processes (9). NDV virus targets and replicates within tumor cells and stimulates host immune response via cytotoxic T lymphocytes and natural killer cells. The virus envelope protein enhances its oncolytic capabilities, and the activation of the apoptotic pathway triggers processes like autophagy, apoptosis, and immunogenic cell death (9). Probiotics are live microorganisms that offer potential health benefits to the host when consumed. They can be classified into lactic and non-lactic acid-producing bacteria and fungi. Examples of probiotics include *Lactobacillus, Lactococcus, Bifidobacterium,* and *Enterococcus* (10). Among these, B.C, a lactic acid-producing probiotic, has numerous functional benefits. It is a Gram-positive bacterium that can synthesize lactic acid and survive in both aerobic and anaerobic conditions (11). Probiotic products, such as cell culture supernatant, exo-polysaccharide extracts, bacterial wall components, and heat-killed bacteria, have been studied (12). The metabolites released in the supernatant of probiotic bacteria have shown potential benefits due to their ease of use, stability, and diverse properties, such as anti-inflammatory, cytotoxic, antioxidant, and immunomodulatory effects against cancer cells (13). This study investigates the combined impact of B.C and NDV on the growth of human colorectal adenocarcinoma cells.

MATERIALS AND METHODS

HT29 cell line. The HT29 (HTB-38TM) cell line was procured from the National Cell Bank of Iran, located in Tehran at the Pasteur Institute of Iran. The cancerous cells were cultured in flasks containing DMEM and 10% FBS. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and 90-95% humidity. Once the cells reached 80% confluence, they were subjected to trypsinization. After cell counting, the cells were seeded into each well of a 96-well plate and were used for further evaluations (14). The study consisted of six groups: Group I: Control (without treatment), Group II: *Newcastle Disease Virus* (NDV), Group III: *Bacillus coagulans* (B.C), Group IV: 5-Fluorouracil (5-FU; 1.44 mM), Group V: *Newcastle disease virus* and *Bacillus coagulans* (NDV+B.C), Group VI: *Newcastle disease virus, Bacillus coagulans* and 5-Fluorouracil (Combined+5-FU).

***Newcastle disease virus* and cytotoxic effects.** The NDV LaSota strain (14) was provided by the Applied Virology Research Center of Baqiyatallah University of Medical Sciences. To determine the multiplicity of infection (MOI) for cell inoculation, the ratio of viral particles to cells was calculated. MOIs of 0.1, 0.5, 1, 2, 4, 8, and 10 were prepared based on cell count, according to the initial virus dose. The cytotoxic effects of the NDV were assessed using the Cell Proliferation Assay Kit (MTT). For this purpose, HT29 cells were seeded in each well of plates at a density of 3.0×10^4 and were grown in the culture plates. The cells were infected with NDV at different MOIs (0.1, 0.5, 1, 2, 4, 8, and 10). After 72 hours of incubation, the medium was replaced with DMEM and MTT (5 mg/ml) solution was added to each well. The plate was then incubated at 37°C for 4 hours. After removing the incuba-

tion medium, dimethyl sulfoxide (DMSO) was added to dissolve the purple crystals of formazan, and the absorbance was measured at 540-570 nm using an absorbance microplate reader. This was done to assess the MTT reduction (Cell Viability (%): Absorbance of treated cells / Absorbance of control cells × 100). The experiment was repeated at least three times. The MOI with the half-maximum inhibitory concentration was determined based on the MTT test results using GraphPad software. The evaluations were then continued using this MOI.

***Bacillus coagulans* (GBI-30, 6068) and cytotoxic effects.** According to the study conducted by Dolati et al. (2021) (11), a bacterium-free culture supernatant was prepared for experimentation. To do this, after the bacterial culture had completed its growth phase, the bacterial culture supernatant was collected and purified by centrifugation at 6000 rpm at room temperature for 20 minutes. The purified supernatant was filtered through a 0.2-micron filter to ensure that there were no bacteria present. Then, it was dried using a freezer dryer and stored in a refrigerator. Different concentrations of the culture supernatant (5, 10, 20, 40, 80, and 100 µg/ml) were prepared for the experiment. To evaluate the cytotoxic effects of the culture supernatant, a Cell Proliferation Assay Kit (MTT) was used. For this purpose, HT29 cells were seeded in each well of the plates at a 5.0×10^3 density and grown in these cell culture plates. The cells were treated with varying strengths of bacterial supernatants. The plates were then incubated for 72 hours. After the medium was removed from the wells, a fresh medium was added. Then, 20 µL of MTT (5 mg/ml) was added in the dark and the mixture was incubated for 4 hours to create formazan crystals. The purple crystals of formazan were dissolved by adding 100 µL of dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 540-570 nm using an absorbance microplate reader to evaluate the MTT reduction. The concentration with half-maximum inhibitory concentration was determined based on the MTT test results, using GraphPad software. The evaluation process will continue at this concentration.

Evaluation of HT29 apoptosis. To determine the apoptosis of HT29 cells, a fluorescent dye consisting of acridine orange (AO) and Propidium Iodide (PI) was used. Single or combined agent treatments were administered to the cells cultured in 24-well plates. After that, the cells were trypsinized, and a fluorescent dye of 10 µl was added to the cellular pellet. The dye contained acridine orange (10 µg/ml) and Propidium Iodide (10 µg/ml) with equal volumes. Then, the dye-cell suspension was placed on a hemocytometer, and examined under a fluorescence microscope. Green cells were considered viable, while red cells were considered apoptotic (15).

Examination of caspase-8 and 9 activity. According to the manufacturer's instructions, and Lee et al. study (2012) colorimetric assay kits (BioVision, UK) was used to measure the activity of caspase-8 and caspase-9 proteins in HT29 cells after they had been exposed to single or combined treatment groups (16).

Statistical analysis. The experiments were repeated three times. The data was analyzed using ANOVA (one-way analysis of variance) in SPSS software (version 22) and GraphPad Prism software 8.0.2. The significance level considered was $P < 0.05$. Data was presented as mean ± standard deviation (SD).

Ethics approval and consent to participate. The experimental protocols for all research conducted were ethically approved by the university's Ethical Committee (approval number: IR.KUMS.MED.REC.1401.092).

RESULTS

Fig. 1 illustrates the proliferation of HT29 cells in a cell culture setting. Once the cells reached 80% confluence, they were detached from the surface of flask and seeded into a 96-well plate. Afterward, the cells were treated with NDV, B.C, and 5-FU, both individually and simultaneously.

IC50 of NDV and *B. coagulans*. Fig. 2 displays that NDV (A) and B.C (B) had IC50 values of approximately 5 MOI and 90 µg/ml, respectively, against HT-29 cells.

Fig. 1. Growth process of HT29 cells in cell culture flask (100X).

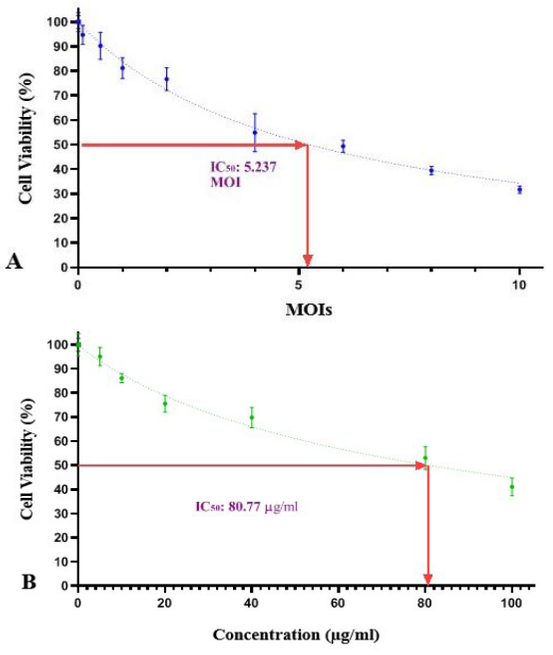

Fig. 2. IC50 of NDV (A) and B.C (B) on HT29 cell line.

Cell cytotoxicity (MTT assay) and viability/apoptosis analysis. Fig. 3A shows that all treatment groups reduced the proliferation of HT29 cells compared to the control group (P<0.0001). Although the rate of decrease in proliferation was higher in the treatment group with 5-FU, there was no significant difference between the treatment group of NDV, B.C and 5-FU (P>0.05). Similarly, no significant difference was observed between the 5-FU group and the NDV+B.C group (P>0.05). However, a significant reduction was observed between the NDV+B.C group and the B.C group P<0.05. Although there was no significant difference between the NDV+B.C group and the NDV group (P>0.05), the intensity of the reduction was greater in the NDV+B.C group. The Combined+5FU group showed the greatest decrease in proliferation and was significantly different from all treatment groups (P<0.0001). It is shown that the survival rate of HT29 cells decreased after all treatments compared to the control group (without treatment) (Fig. 3B). The combination of NDV and B.C had an apoptotic effect equivalent to that of 5-FU, indicating the significant impact of this therapy. Our study highlights the enhanced efficacy and synergistic benefits of using 5-FU in combination with NDV and B.C, which outperforms the effects of 5-FU administered alone.

Caspase activity assay. Following a 72-hour incubation period with NDV and B.C, there was a significant

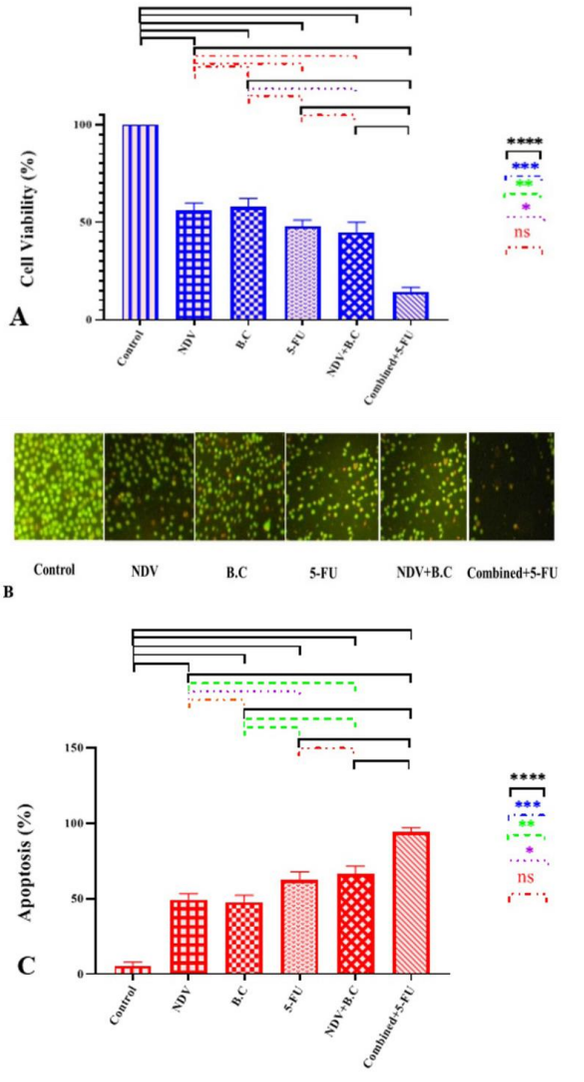

Fig. 3. Effects of NDV, B.C, and 5-FU treatments on HT29 cell line cytotoxicity (A), apoptosis image (B), and apoptosis percentage (C) (* P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001)

rise in the levels of caspase-9, as demonstrated in Fig. 4. No significant increase was observed in caspase-8 levels of the cells treated with NDV and B.C. These findings suggest that both NDV and B.C play a role in triggering apoptosis by activating intrinsic apoptosis pathway.

DISCUSSION

This study investigated the combined impact of B.C and NDV on human colorectal adenocarcinoma cells. The results showed that the application of NDV

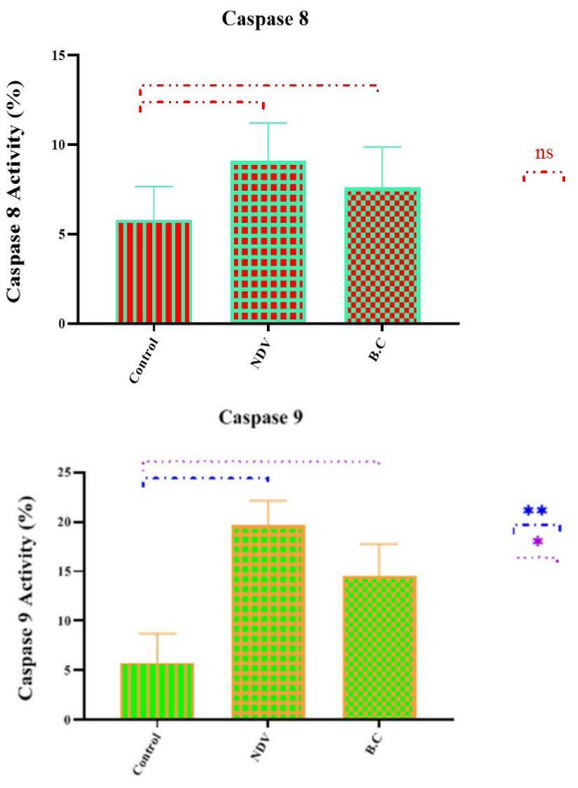

Fig. 4. The effects of NDV, B.C, and 5-FU treatment on caspase 8 and caspase 9 activity of HT29 cell line (* P<0.05, ** P<0.01, *** P<0.001 and **** P<0.0001).

and B.C led to a significant increase in cell death and a reduction in cell growth, and the combined use with 5-FU exhibited a synergistic effect in decreasing cell proliferation. Intrinsic apoptosis pathway was activated by B.C and NDV. Studies show that probiotics and OVs offer promising treatment options for cancer. NDV infects birds without harming humans, yet it infects and replicates within human cancer cells while sparing normal cells (17). Injecting NDV into tumors triggers immune responses, enhances inflammation, and attracts tumor-specific T cells. Combining immune checkpoint blockade with viruses encoding immunomodulatory ligands can further enhance these effects (18). NDV OVs are effective against various cancer cell lines due to the virus inherent oncolytic capacity and host cancer cell susceptibility. Hemagglutinin-neuraminidase (HN) and fusion (F) proteins play a crucial role in inducing oncolytic effects by facilitating the formation of syncytia (19, 20). Probiotics are microorganisms that provide health benefits. B.C is a beneficial *Bacillus* species that was isolated from contaminated canned milk in 1915. B.C strains were misidentified as *Lactobacillus sporogenous*, but subsequent research revealed its potential in treating cattle, aquaculture, and human GIT disorders. Notably, it has shown promise in addressing IBS, antibiotic-associated diarrhea, inflammatory bowel disease, and colorectal cancer. These conditions were the primary focus of animal and preclinical studies involving B.C. (21, 22). In 2019, Keshavarz et al. reported that the viability of TC-1 cell lines was significantly reduced through apoptotic cell death by NDV treatment (23). Similarly, Mansour et al. observed an increase in NDV replication and syncytia formation in A549 tumor cells, leading to apoptosis in 2011 (24). Furthermore, a recent study by Jalali Kondori et al. in 2022 showed that combining the Newcastle oncolytic virus with copper nanoparticles, hyperthermia, and radiation had cytotoxic and apoptotic synergistic effects, especially on colon cancer cell lines (25). Multiple studies, including those by Meng et al. in 2020 (26) and Syed Najmuddin et al. in 2020 (27), have consistently found that the NDV significantly decreases cancer cell survival while promoting apoptosis. The NDV has multiple anticancer mechanisms. It triggers cell death through internal and external receptors, and increases caspase-related activities. It also uses the hemagglutinin-neuraminidase protein to enhance its effects. Studies suggest that activating TRAIL and caspase pathways plays a crucial role in inducing apoptosis in HeLa cells. Additionally, the primary driver of apoptosis in NDV-infected Vero cell line is the mitochondrial intrinsic apoptotic pathway (28). Our research results demonstrate that NDV effectively utilizes intrinsic pathway to initiate apoptosis. Several studies have reported on the inhibitory effects of supernatants from various probiotic strains on the growth of cancer cells. For example, Dehghani et al. found that *Lactobacillus rhamnosus* supernatants can inhibit the growth of HT29 cells and induce apoptosis in a dose- and time-dependent manner (29). Similarly, Nami et al. found that *Enterococcus lactis* IW5 supernatants have inhibitory effects on the growth of multiple cancer cell lines, including MCF7, Hela, AGS, HT29, and Caco-2, while showing no toxicity towards FHs-74 normal cells (30). Another study by Madempudi et al. demonstrated that B.C has anti-cancer properties against Hela, COLO 205, and K562 cell lines, while not affecting the proliferation of healthy HEK 293T cells (31). B.C has cytotoxic effects on breast cancer cells according to Dolati et al. (2021). Findings showed upregulated

genes, caspase 3, caspase 9, and Bax, and downregulated anti-apoptotic gene, Bcl-2, leading to cancer cell apoptosis (11). Elankumaran et al. reported that NDV induced apoptosis in Caco-2 and HT29 cells without activating caspase-8 (32). Our investigations also discovered B.C cytotoxic and apoptotic effects on the HT29 cell line via intrinsic pathway, making it a promising anti-cancer agent.

CONCLUSION

CRC is a significant global health challenge, requiring innovative approaches for effective treatment. Our results revealed that the combination of NDV and B.C promise in impeding the growth of human colorectal adenocarcinoma cells with reduced side effects. NDV selectively targets and induces apoptosis in cancer cells while B.C produces lactic acid that has remarkable cytotoxic and apoptotic effects on cancer cells. When combined with 5-FU chemotherapy drug, NDV and B.C exhibited a synergistic suppression of cancer cell proliferation, suggesting a promising avenue for developing more effective and less toxic CRC treatments.

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