

## The Oncolytic Effect of an Iraqi Newcastle Disease Virus Attenuated Strain (AMHA1) Against Colorectal Cancer Cells

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### Abstract

**Background:** The most recent World Health Organization statistics show that colorectal cancer is the third foremost cause of death by cancer among Iraqis. It has become necessary to search and develop novel and unconventional treatment methods. Oncolytic virotherapy one of the innovative cancer therapeutics that showed to be safe and selective and there is increased efforts in the recent years to move them to clinic. For this reason the development of Iraqi strain oncolytic Newcastle disease virus is very important to fight colorectal cancer. **Objective:** The study aimed to study the oncolytic effect of Newcastle disease virus on colorectal cancer cell. **Materials and Methods:** This study tested the killing rate of Iraqi strain of Newcastle disease virus (AMHA1) on colorectal cancer cells and testing the safety on normal cells. The virus tittered using Vero cells, and then the cytotoxic effect of the virus on cancer cells and normal cells was determined using the crystal violet assay. Finally, we performed a morphological analysis using hematoxylin and eosin staining. **Results:** Newcastle disease virus 0.3 MOI causes significant cytotoxicity with prominent cytopathic effect in the colorectal cancer cell line HRT-18G with 50% percentage of growth inhibition, but not in the normal human fibroblast (NHF) percentage of growth inhibition where it was less than 20%. **Conclusion:** oncolytic NDV AMHA1 strain effective against colorectal cancer cells because it has selectivity in replication and safety profile.

**Keywords:** oncolytic virotherapy, Newcastle Disease Virus, colorectal cancer cell line.

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## 1. Introduction:

Colorectal cancer (CRC) is one of the world's leading causes of death. The conventional therapy of CRC is surgery and a treatment regimen of chemotherapy, radiotherapy, and immunotherapy; all have limitations, such as tumor drug resistance and severe side effects (1). Oncolytic virotherapy is a considerably promising technological advancement in cancer gene therapy (2). It uses viruses that infect and lyse tumor cells without hurting normal cells. Newcastle disease virus (NDV) is a wild oncolytic virus. This virus is classified as a member of the family Paramyxoviridae (3). It is a virus with an envelope and a single-stranded negative-sense RNA (-SSRNA) genome. Six RNA genes encode several proteins. The most important proteins for virus entrance are the Hemagglutinin-Neuraminidase (HN) protein and the Fusion protein (F). HN and F are the two main surface spike proteins. The HN protein mediates virus binding, whereas the F protein permits the virus's envelope to merge with the plasma lemma of the target cell (4). This membrane fusion permits the viral genome to enter the host cell's cytosol. The negative SSRNA is then translated into viral proteins after being transcribed into messenger RNAs (positive SSRNA) in the cytoplasm of the infected cell, and this is one of the important criteria of oncolytic viruses that should not integrate with the host cell genome (5). When an oncolytic virus infects normal cells, the production of double-stranded RNA (dsRNA) is critical to the life cycle of all oncolytic viruses because it initiates various cellular defense mechanisms involving the production of IFNs and many molecular chaperones. Tumors provide a moderately hospitable environment for spreading RNA viruses like NDV because tumor cell mutations frequently disrupt the IFN operation system. The initial steps of NDV infection occur in all cell types, but the viral replication step occurs primarily in malignant cells because it is arrested in normal cells (6). The current study aimed to investigate the oncolytic activity of an Iraqi Newcastle Disease Virus Attenuated Strain (AMHA1) against colorectal cancer.

## 1. Materials and Methods:

### 1.1 Virus and Cell lines:

Iraqi attenuated strain of Newcastle disease virus (AMHA1) was provided by Prof. Dr. Ahmed Majeed Al-Shammari lab. Vero cell line is one of the most commonly used mammalian continuous cell lines in virology and derived from the kidney of an African green monkey used just for virus titration TCID<sub>50</sub>. Normal human fibroblast cell line (NHF) this is an Iraqi cell line established by Prof. Dr. Ahmed Majeed Al-Shammari produced from a biopsy specimen of human adipose tissue, and human colorectal adenocarcinoma cell line (HRT-18G) were provided by Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University.

### 1.2 Preparation of Media and Stain Used for Cell Culture Experiments:

All tissue culture solutions prepared as manufacturer company method except Phosphate buffer saline was ready to use (PBS) (Sigma-Aldrich, USA) and Hematoxyline and Eosin were ready to use (H&E) (Syrbio, SAR).

**1.2.1 Minimum Essential Medium (MEM) (US Biological, USA), Complete – Growth Medium 10%FCS-MEM.** MEM culture medium was prepared by dissolving 16.65 g MEM powder supplemented with 4-(2-hydroxyethyl)-1piperazineethane sulfonic acid (HEPES) and L-glutamine in approximately 800 mL of deionized water with gentle stirring until completely solubilized. The medium was completed by adding the following ingredients: Sodium Bicarbonate (2.2 g), Fetal Calf Serum (Capricorn, Germany) (100 ml), and Antibiotic/Antimycotic Solution (Capricorn, Germany) (100X) (10 mL). pH was adjusted to the 7.2, and then the medium volume was completed to 1 L. The medium was sterilized by using syringe filter, 0.22 µm filter unit. Finally, the media was aliquot into sterile containers, stored at 4°C and used within a short period of time.

**1.2.2 Maintenance Serum Free Medium (SFM-MEM).** Maintenance serum free medium is MEM excluded from fetal calf serum.

**1.2.3 Trypsin-EDTA Solution (US Biological, USA).** Trypsin-EDTA solution was prepared by dissolving 10.1 g of trypsin-EDTA powder in one liter of deionized water. The solution was stirred constantly on a magnetic stirrer at room temperature and sterilized by syringe filters, pore size 0.22 µm. The solution was stored in refrigerator and used within a short period of time.

**1.2.4 Crystal Violet Solution (Santa Cruz, USA).** The solution was prepared by suspending 0.5 g of crystal violet stain powder in 100 mL of 20% methanol. The solution was filtrated by using Whatman No.1 filter paper and kept in amber glass container at ambient temperature and used within two months(7).

**1.3 Maintenance of *in vitro* Cell Lines:**

Cell lines (Vero, HRT-18G and NHF) were sub-cultured in cell culture flasks (25 cm<sup>2</sup>) using complete growth MEM. When cell growth exceeding 80% confluency, the growth medium was decanted off and the cell washed three times with PBS. After washing, 2-3 mL of trypsin-EDTA solution was added to the flask and the flask was turned over to cover the monolayer completely with gentle shaking. The flask was incubated at 37°C for 1-2 min until the cells were detached from the surface of the flask. Cells were dispensed in 10% FCS- MEM and then redistributed at the required concentration into culture flasks and incubated at 37°C supplemented with 5% CO<sub>2</sub> (8).

**1.4 Newcastle Disease Virus (NDV) tissue culture infected dose (TCID<sub>50</sub>):**

Virus was titrated on Vero cells and the tissue-culture infectious dose 50 (TCID<sub>50</sub>) was calculated depending on principles of Karber and Spearman. The assay was achieved by using 96-well flat-bottomed micro titration plates as previously described Sharma *et al.*, 2016 (9). When reached confluent monolayer, Vero cells were detached from culture flask by trypsinization, then 20 mL of complete growth MEM medium were added to the flask and mixed gently to prepare cell suspension. Aliquot of 200 µL of cell suspension was transferred to each well in micro titration plate (each well contain approximately 7 x 10<sup>3</sup> cell/well). The plate was covered with a sterile adhesive film, gently shook for few min and incubated at 37°C, 5% CO<sub>2</sub> for 24 hrs. Preparation of Diluted NDV by ten sterile Eppendorf tubes (1.5 mL) were formatted and numbered in sequence; all tubes were fixed on ice pack during the experiment. Cold SFM was prepared and 900 µL of SFM was added in sequence to all Eppendorf tubes. Aliquot of 100 µL of virus stock suspension was added serially tenfold diluted after replacing the tip each time. Exposure Stage to calculate TCID<sub>50</sub>, the amount of a pathogenic agent that will cause infection in 50% of cell cultures inoculated, was detected according to the following procedure. Vero cells were seeded at 7000 cell/well in 96-well microplate flat bottom, and after 24 hrs or when confluent monolayer was achieved, growth media was discarded and virus suspension (ten-fold serial dilution) was prepared, 1/10, 1/10<sup>2</sup>, 1/10<sup>3</sup>, 1/10<sup>4</sup>, 1/10<sup>5</sup>, 1/10<sup>6</sup>, 1/10<sup>7</sup>, 1/10<sup>8</sup>, 1/10<sup>9</sup>, and 1/10<sup>10</sup>. Then, the cells were inoculated with 50 µL/well of NDV with 6 replicates for each dilution. Cells without treatment considered control. The plate was covered with a sterile adhesive paper and incubated for 2 hrs at room temperature to allow virus penetration. The plates were re-incubated at 37°C for 72 hrs or until the syncytia observed by inverted light microscope. After incubation, the plate was stained with crystal violet solution (50µL/well) and incubated for 20 min in dark environment, then washed with ddH<sub>2</sub>O. The plate was allowed to dry to calculate the TCID<sub>50</sub>. Any well with syncytia or cytopathic effect (CPE) is considered a positive result (10). Fig1A. The formation of syncytia after 72 hrs of virus infection in each well was marked positive and virus titer was calculated by using the following equation:

$$\log_{10}(\text{TCID}_{50}/\text{mL}) = L + d(s-0.5) + \log(1/v)$$

L = negative log<sub>10</sub> of the most concentrated virus dilution tested in which all wells are positive.

d = log<sub>10</sub> dilution factor.

s = sum of individual pi “proportion of an individual dilution”.

pi = calculation proportion of an individual dilution.

(Amount of positive wells/total amount of wells per dilution)

v = volume of inoculum (mL/well).

**2.5 Cytotoxicity of NDV against HRT-18G and NHF Cell Lines:**

The cells (7 x 10<sup>3</sup>cell/well) were grown in 96 flat well micro-titer plates, in a final volume of 200µl complete culture medium per each well. The microplate was covered by sterilized parafilm, gently mixed, and incubated for 24 hrs at 37°C, 5% CO<sub>2</sub>. Viral Exposure done by removing medium aseptically. Then the cells were exposed to a range of multiplicity of infections (MOI) of NDV (0.1, 0.5, 1, 3, 5, 10, and 20) by adding 100 µL of each MOI of NDV to each well (Table1). Six replicates were used for each MOI while control cells left without treatment.

**Table (1): Preparation of different viral MOI depending on TCID<sub>50</sub>.**

MOI/NDV	No. of NDV/10 Cells	No. of NDV/7000 Cells (1 well)	Volume of NDV $\mu$ L/well	Volume of NDV $\mu$ L/6 wells
0.1	1	700	0.018	0.1
0.5	5	3500	0.093	0.558
1	10	7000	0.186	1.116
3	30	21000	0.558	3.348
5	50	35000	0.93	5.58
10	100	70000	1.86	11.16
20	200	140000	3.72	22.32

**MOI: multiplicity of infections; NDV: Newcastle Disease Virus**

NDV was added for 2 hrs at room temperature with shaking to allow virus attachment and penetration. After that, the solution was removed immediately from each well using micropipette. Cells were washed with PBS and 0.2 mL of pre-warmed SFM was added. The plate was covered again with a new sterile adhesive film and sealed with parafilm. The plates were re-incubated at 37°C for three days or until the syncytia were observed (11). After 72 h of incubation at 37C, Add 50  $\mu$ L of 0.5% crystal violet staining solution to each well, and incubate for 20 min at room temperature. Wash the plate four times with tap water. After washing, invert the plate on filter paper and tap the plate gently to remove any remaining liquid. Air-dry the plate without its lid for at least 2 h at room temperature. Add 200  $\mu$ L of methanol to each well, and incubate the plate with its lid on for 20 min at room temperature. Measure the optical density of each well at 570 nm (OD570) with a plate reader. Subtract the average OD570 of the wells without cells from the OD570 of each well on the plate. Set the average OD570 of nonstimulated cells to 100%. Then determine the percentage of stimulated cells that are viable (attached) by comparing the average OD570 values of stimulated cells with the OD570 values of the non stimulated cells. Calculate the mean and the standard error of the mean for three independent experiments. Steps done according to the method of Feoktistova *et al.* fig.1B (7) ;(12).

Finally, study the morphological changes under a light microscope by staining with hematoxylin and eosin (H&E) according to the method of Silva and his coworker fig1C (13).

## 2.6 Statistical Analysis:

Student's *t*-test was performed to test whether group variance was significant. Statistical significance was defined as \*  $p \leq 0.05$  or \*\*  $p \leq 0.01$ . All samples analyzed statistically were run in triplicates (unless mentioned otherwise). Data were expressed as mean  $\pm$  standard deviation, and statistical significances were carried out using Graph Pad Prism version 8 (Graph Pad Software Inc., La Jolla, CA).

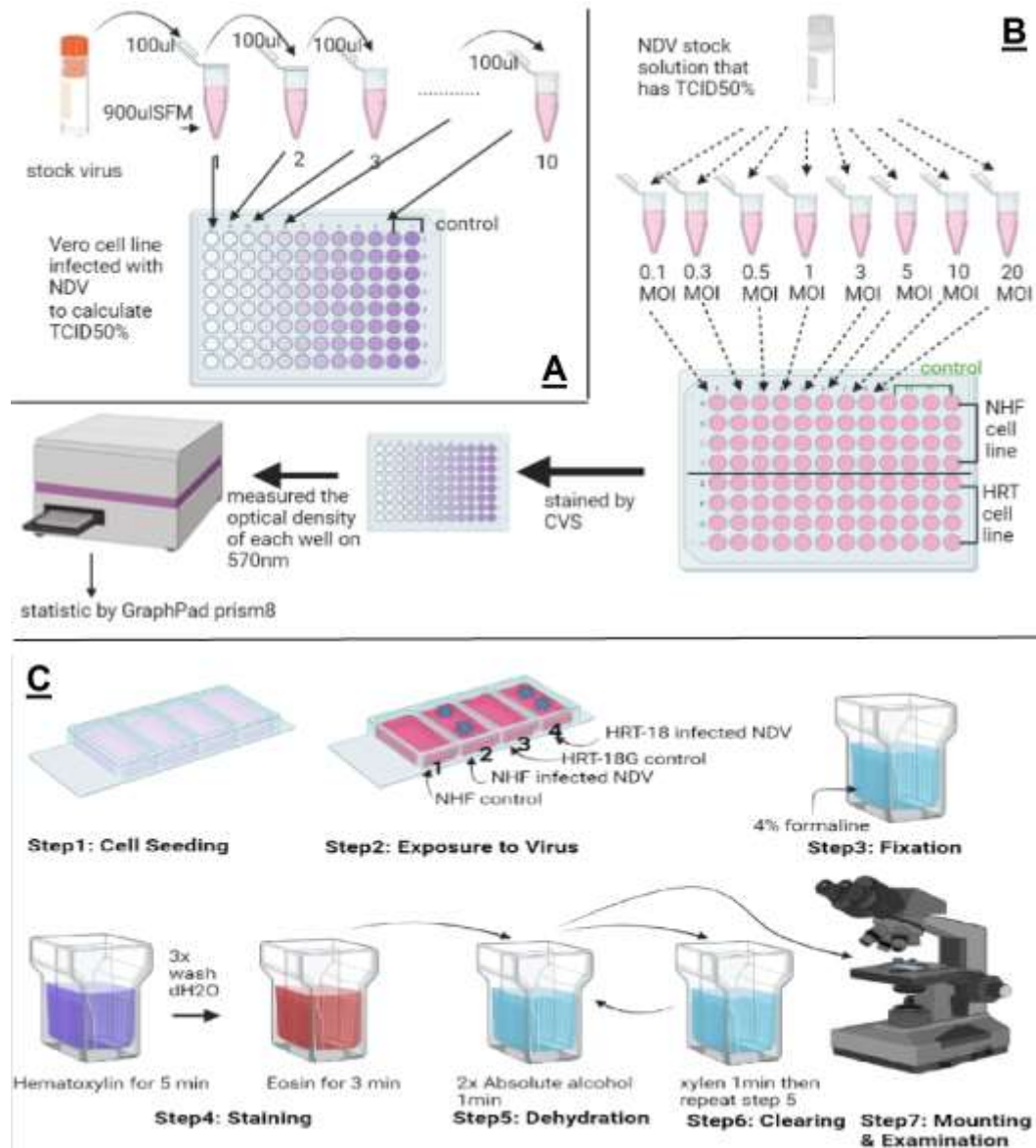


Figure (1): Study Design, A. method of virus titration TCID<sub>50</sub>% on Vero cell line. B. illustrates the IC<sub>50</sub> calculation method on NHF and HRT-18G; C. morphological study by H & E. Figure is created with BioRender.com

### 3. Results:

#### 3.1 Tissue Culture Infectious Dose (TCID<sub>50</sub>) of NDV:

The NDV titer was measured on the Vero cell line to determine the TCID<sub>50</sub> of the virus. The average measurement of TCID<sub>50</sub> was 39.0 x 10<sup>6</sup> virus mL<sup>-1</sup> after three independent experiments (Table2).

**Table (2): Average calculation of NDV TCID<sub>50</sub> using Vero cells. The exposure was carried out for 72 hrs.**

Experiment 1			Experiment 2			Experiment 3		
log10 Virus Dilution	Ratio of Infection	pi	log10 Virus Dilution	Ratio of Infection	pi	log10 Virus Dilution	Ratio of Infection	pi
-1	6/6	1	-1	6/6	1	-1	6/6	1
-2	6/6	1	-2	6/6	1	-2	6/6	1
-3	6/6	1	-3	6/6	1	-3	6/6	1
-4	6/6	1	-4	5/6	0.83	-4	6/6	1
-5	5/6	0.83	-5	3/6	0.5	-5	5/6	0.83
-6	3/6	0.5	-6	3/6	0.5	-6	3/6	0.5
-7	0/6	0	-7	2/6	0.33	-7	0/6	0
-8	0/6	0	-8	0/6	0	-8	0/6	0
-9	0/6	0	-9	0/6	0	-9	0/6	0
-10	0/6	0	-10	0/6	0	-10	0/6	0
TCID <sub>50</sub> 1.3 x 10 <sup>7</sup>			TCID <sub>50</sub> 9.1 x 10 <sup>7</sup>			TCID <sub>50</sub> 1.3 x 10 <sup>7</sup>		
Average TCID <sub>50</sub> 3.9 x 10 <sup>7</sup> Virus mL <sup>-1</sup>								

**pi: Proportion; TCID<sub>50</sub>: Tissue Culture Infectious Dose 50%; NDV: Newcastle Disease Virus.**

### 3.2 CVS cytotoxicity assay:

The CVS cytotoxicity assay was used to calculate the cytotoxicity percentage at various NDV treatments (0.1, 0.3, 0.5, 1, 3, 5, 10, and 20 MOI). Results show that the viability of HRT-18G was dramatically decreased by increasing the NDV MOI with the inhibition rate of HRT-18G cells ranging from 43.9±3.9 to 83.16±2.7%. All applied doses of viral MOI were significantly ( $p < 0.01$ ) displaying a killing effect against HRT-18G cells compared to the normal cells of NHF cell line, with the substantial effect of NDV being greater in inhibiting colon cancer cells than in normal cells. Fig. 2.

### 3.3 Morphological changes in colorectal cancer cells infected with NDV:

Colorectal cancer cells undergo morphological changes that occurred after 72 hours of 0.3 MOI NDV infections were contributed to the NDV-induced cytopathic effect. Only NDV-infected cells showed significant cytopathic effect as demonstrated in figure 3 and 4, a large number of cramped cells with granulation, cell detachment, rounding, vacuolation, multinucleated giant cell formation and shrinkage of cells which results in opaque foci of cells which leads to separation and floating of the treated cells in the cultured media. And also can show the empty plaque spaces between cells compared with the control.

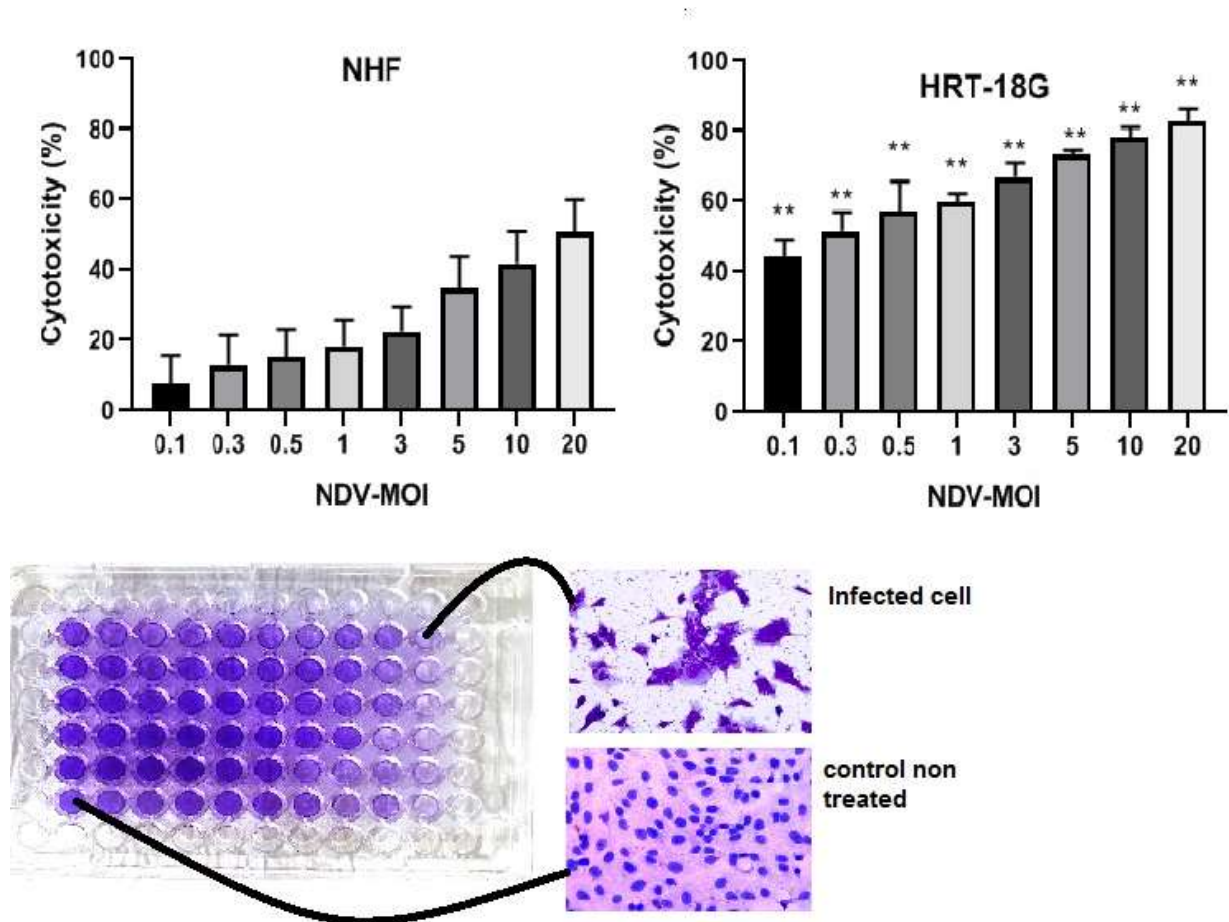


Figure (2): NDV induced significant cytotoxicity in colorectal cancer cells (HRT-18G). After 72 hrs of incubation at 37°C. \*\*: p-value  $\leq 0.01$ ; NDV: Newcastle Disease Virus; MOI: Multiplicities of Infection.

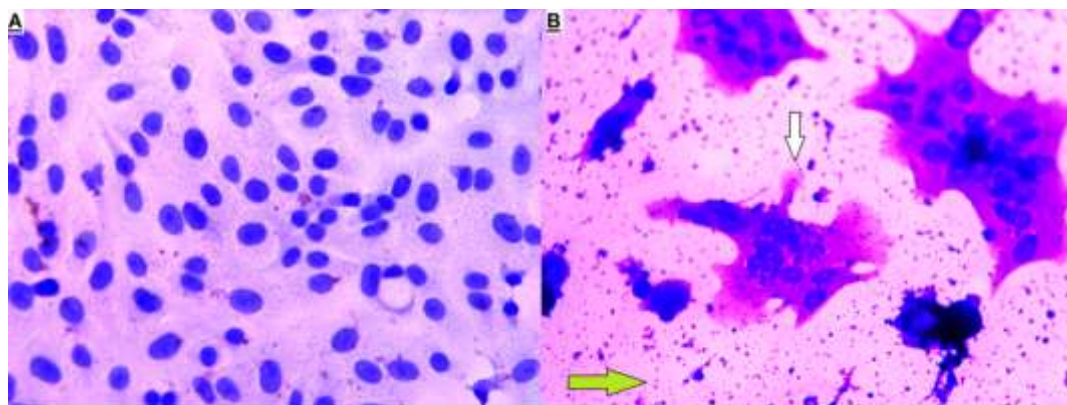
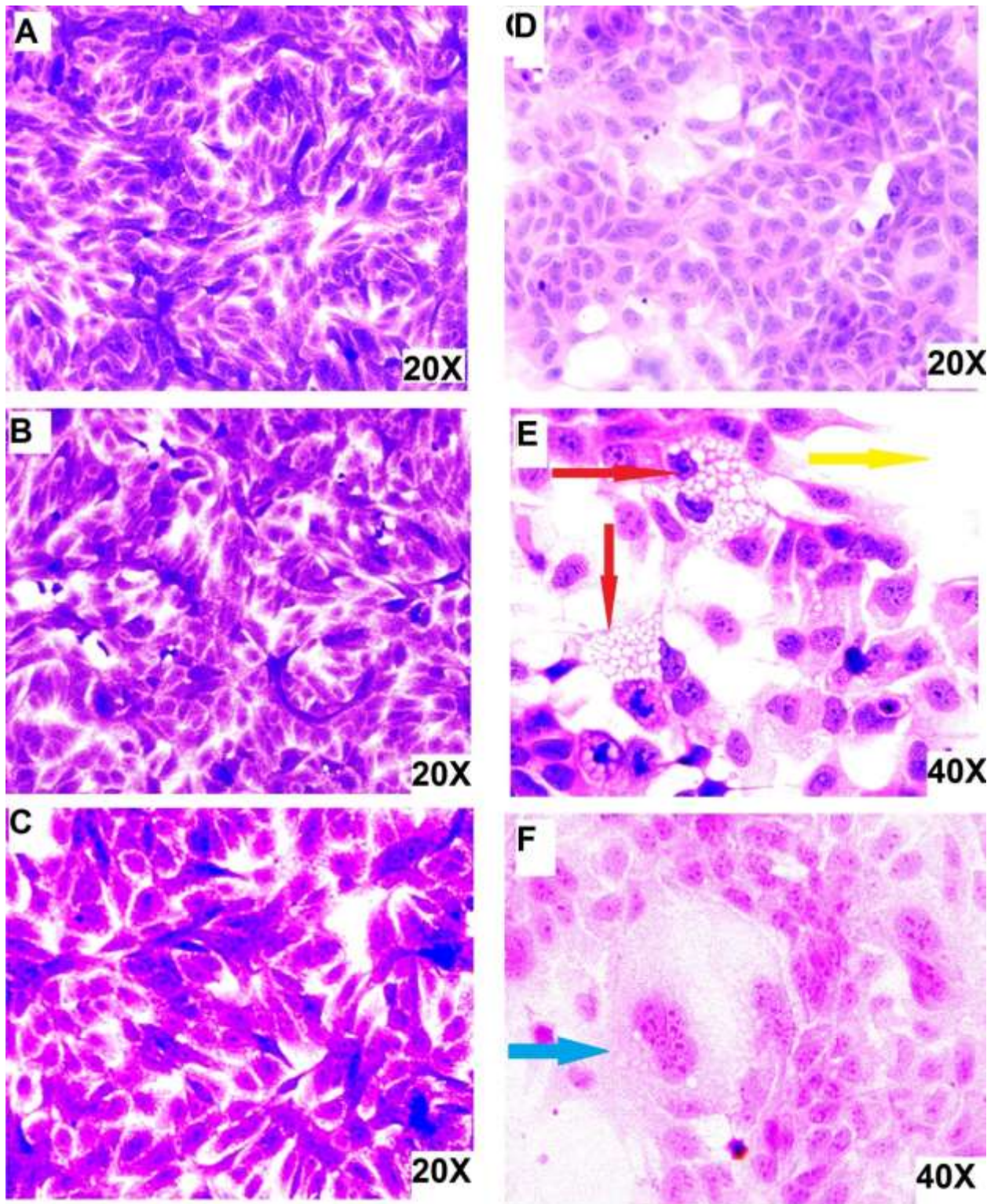


Figure (3): the cytopathic effect of NDV in HRT-18G cell line after 72 h of incubation and stained with crystal violet. A. cells none infected with NDV (control). B. cells infected with 0.3 MOI NDV, the white arrow represents the cytoplasm blebbing, and the green arrow represents the empty plaque spaces between cells.



**Figure (4):** Morphological Study of NHF (left lane) and HRT-18G (right lane) cell lines treated with NDV and stained with H & E. A and D are the untreated control cells. B and E cells are infected with NDV for 42 hours, and C and F for 72 hours. The red arrow represents the vacuolation, the blue arrow represents the syncytia, and the yellow arrow represents the empty plaque spaces between cells.



### **Discussion:**

The current project was designed to find novel therapy for one of the most difficult tumors which is colorectal cancer. Colorectal cancer still is the most frequent cancer of the gastrointestinal tract throughout the world (14). The incidence of CRC has been increasing rapidly, and it has been expected that 2.4 million new cases will be detected yearly worldwide by 2035. Because of some side effects and complications of traditional CRC therapies (15), investigated oncolytic virotherapy that made great progress in tumor therapy due to the efficient killing effect of some viruses, which can infect, propagate, and lyse cancer cells while ignoring normal cells. Oncolytic viruses replicate selectively in the cytosol of tumor cells and are not integrated into the host genome, making them safer, effective, and appealing as oncolytic agents (16).

This selectivity and efficiency of the oncolytic virus is based on important mechanisms. The first one is receptor-mediated uptake of the virus in cancer cells more than normal, especially since many CD markers are overexpressed in cancer cells like CD 49, and the virus uses it as a receptor for entry. The second mechanism is virus adaptation to host cancer signaling pathways for viral reproduction. While normal cells have strong interferon signaling, it results in preventing virus replication (17) ;(18). The third one is tumor cell death via apoptosis induction. The fourth mechanism is that virus promotes acquired immunity (anti-tumor response) by enhancing tumor antigen presentation in virus-infected tumor cells (19).

The results show that the NDV (AMHA1) strain has oncolytic properties against CRC cells when compared to normal cells. The virus had no harmful effect on human normal cells, confirming virus safety, as there was no difference in cell number between control and NDV-treated cells. However, when HRT-18G cells were exposed to NDV AMHA1, they died significantly more than untreated control cells. Because of the oncogenic transformation, cancer cells are sensitive to virus infection for several reasons, one of which is the defect in the interferon pathway (20). NDV AMHA1 strain-induced cytopathic effect in infected HRT-18G cancer cells morphology or the shape of cells included cell rounding, cytoplasmic vacuolation, syncytia formation, and cell-to-cell fusion caused by the fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins on the NDV surface, leading eventually to cell death. NDV infection of the normal NHF cells showed no such killing effect. Previous studies showed that oncolytic NDV can induce nuclear condensation, cytoplasmic blebbing, DNA fragmentation, cell-cell fusion, and the formation of mononuclear cell aggregates (syncytia) (21) ;(22).

### **Conclusion:**

NDV is a promising oncolytic virus because it has selectivity in replication, safety profile, anticancer activity, and immunostimulatory features.

### **Financial support and sponsorship**

No financial support.

### **Conflicts of interest**

There are no conflicts of interest.

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## التأثير الحاد للسلالة المضعفة من فيروس نيوكاسل العراقي (AMHA1) ضد خلايا سرطان القولون والمستقيم

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### الخلاصة:

**خلفية عن الموضوع:** تظهر أحدث إحصائيات منظمة الصحة العالمية ان سرطان القولون والمستقيم هو ثالث أهم أسباب الوفاة بسبب السرطان بين العراقيين، أصبح من الضروري البحث عن طرق علاج جديدة وغير تقليدية وتطويرها. علاج الأورام الفيروسي هو أحد علاجات السرطان المبتكرة التي أظهرت أنها آمنة واثقائية ، وهناك جهود متزايدة في السنوات الأخيرة لنقل هذه العلاجات إلى التجارب السريرية. لهذا السبب فإن تطور فيروس مرض نيوكاسل المسبب للأورام في العراق مهم جدا لمحاربة سرطان القولون والمستقيم. **الهدف من البحث :** هدفت الدراسة إلى دراسة تأثير حال الورم لفيروس مرض نيوكاسل على خلايا سرطان القولون والمستقيم. **المواد وطرق العمل:** اختبرت هذه الدراسة معدل قتل السلالة العراقية من فيروس نيوكاسل (AMHA1) على خلايا سرطان القولون والمستقيم واختبار السلامة على الخلايا السليمة. انتشر الفيروس باستخدام خلايا فيرو ، ثم تم تحديد التأثير السام للخلايا للفيروس على الخلايا السرطانية والخلايا الطبيعية باستخدام مقايسة البنفسج البلوري. أخيراً ، أجرينا تحليلاً مورفولوجياً باستخدام صبغة الهيماتوكسيلين والايوزين. **النتائج:** فيروس مرض نيوكاسل moi (0.3) يسبب سمية خلوية كبيرة مع تأثير اعتلال خلوي بارز في خط خلايا سرطان القولون والمستقيم HRT-18G مع 50 ٪ من تثبيط النمو ، ولكن في الخلايا الليفية البشرية الطبيعية (NHF) لم يكن التأثير بارز حيث كانت أقل من 20٪ نسبة تثبيط النمو. **الاستنتاج:** أن سلالة (AMHA1) العراقية لفيروس النيوكاسل الحالة للورم فعالة ضد خلايا سرطان القولون والمستقيم لأنها تتميز بالانقائية في التكاثر وامنة على الخلايا الطبيعية.

**الكلمات المفتاحية:** العلاج الفيروسي الحال الورم ، فيروس نيوكاسل AMHA1 ، خط خلايا سرطان القولون والمستقيم.