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Oncolytic NDV-AMHA1 Strain Effective against Glioblastoma Cancer Cells

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Abstract

Glioblastoma multiforme (GBM) is one of the most life-threatening types of cancer that is difficult to treat. The search for effective yet safe therapy is progressing and non-conventional therapies such as using viruses as a smart and selective agent against cancer are promising. The aim of the study was the presence of a reliable method to use Newcastle disease virus (NDV) as an oncolytic agent against GBM, which attempted to propagate the NDV in laboratory experiments. Ahmed Majeed Hamza Al-Shammari-1 (AMHA1) attenuated strain of NDV was propagated in chicken embryos. The virus's tittered in Vero-slamed cells to determine the infective dose. MTT cell viability assay was used to investigate the killing effects of NDV on Ahmed-Majeed-Glioblastoma-Multiforme-2005 (AMGM5) human glioblastoma cancer cells derived from Iraqi patients. The infected cells' morphology was studied to measure the cytolytic effect of the NDV in cancer cells. Results showed that After 24 to 72 hours of inoculation, all of the chicken embryos died when the AMHA1 Iraqi NDV strain was injected. Cell viability assay showed that the NDV-AMHA1 strain has cytotoxicity at MOI of 0.1, 0.5, and 1 for 72 hours of exposure to cancer cells. The morphological analysis showed that NDV induces cell death in the infected cells with both necrotic and apoptotic features. In conclusion, the study focuses on the propagation of the oncolytic NDV as a biological agent capable of overcoming treatment resistance through infecting and replicating inside cancer.

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1. Introduction

Cancer is a worldwide issue; this disease does not impact just one organ, but rather spreads across the entire body. According to the International Agency for Research on Cancer's (IARC) most recent study, cancer diagnoses have risen to 8.8 million per year, with the rate anticipated to rise to 13.2 million by 2030 [1]. It is a lethal disease with no geographical or organ limitations this results in a worldwide annual mortality rate of 2 % [2]. Investigating the survival of patients with various types of malignancies, as well as analyzing numerous prognostic factors and treatment procedures that have a substantial impact on their survival, is important because it demonstrates the role of race, economy, and medical system ability in cancer control[3]. Traditional treatments are utilized in a variety of ways: Chemotherapy, surgery, and radiation therapy were created to treat various cancer kinds; however, normal cells were also will affected, damaged, and died as a result of these treatments [4] or a mixture of them[5]. Glioblastoma multiforme (GBM) is the most common and dangerous type of primary brain tumor. With a yearly incidence of two to three people per million. It is responsible for more than half of all primary intracranial tumors [6]. Surgical removal is the standard treatment, followed by radiation and chemotherapy [7, 8]. The recurrence rate is considerable, with a median survival of little over one year, making GBM one of the most difficult tumors to manage [9]. The conventional treatment includes resection, followed by radiation and Temozolomide (TMZ) chemotherapy [10]. Despite this aggressive approach, GBM patients have a median life expectancy of about 15 months [11], and therapeutic response after recurrence is limited [12]. Theoretically, viral treatments work against these traits by directly destroying tumors and inducing anticancer inflammatory responses that get through the GBM Microenvironments and immunosuppressive high-grade glioma (HGG) [13]. Only 5–10% of patients live for more than 5 years [14]. With such a poor prognosis, new therapeutic options for GBM are urgently needed. Virotherapy is a type of cancer treatment that employs replicative-competent oncolytic viruses to kill tumor cells [15]. Oncolytic virotherapy (O.V.) is a type of immunotherapy in which viruses, either naturally occurring or genetically produced, preferentially multiply in malignant cells and kill them. O.V. uses the capabilities of human viruses to cause cancer cells to die naturally [16]. Many genetically modified O.V.with better tumor targeting, antitumor efficacy, and safety have been produced, and some have been tested in clinical trials [17]. The phenomenon of viral oncolysis was found in the 1960s, and a quest for a virus that would be most suitable for clinical use in cancer patients began. In 1965, William A. Cassel reported on NDV as an anticancer agent in humans [18]. The Newcastle disease virus (NDV) has been used to treat cancer patients in the United States and Europe since then [19, 20]. Then, several results in both human clinical research and mice models demonstrated the potential for employing NDV as a therapeutic agent against cancer [21]. NDV is a paramyxoviridae virus belonging to the Avulavirus genus. This viral family consists of enveloped, non-segmented, negative-sense RNA viruses that inflame the respiratory and gastrointestinal tracts of a variety of chicken species [22]. NDV strains are classified as lentogenic, mesogenic, and velogenic in hens based on their pathogenicity [23]. It causes extremely contagious infections in birds of numerous species throughout the world, resulting in enormous economic damage [24]. NDV has already been shown to have potential anticancer activity in lab animals while also being greatly safe [25]. Both intrinsic and extrinsic mechanisms are used by the NDV to induce apoptosis in cancer cells [26]. NDV strains were investigated as an anticancer agent in vivo and in vitro on a variety of cancer cell lines, including brain tumor, leukemia, and breast cancer cell lines [27]. The attenuated strain of (AMHA1) is a naturally oncolytic NDV with extensive anticancer activity [28]. NDV's replication has been evaluated on a variety of human malignancies [29]. After the cancer cell is infected with NDV, it multiplies swiftly and infects neighboring tumor cells by producing the child virions, which are evident three hours after inoculation and form plaques after two days [30]. NDV possesses oncolytic properties and can multiply in tumor cells of humans faster than normal cells, which has piqued the curiosity of researchers [31]. NDV genome has six genes that code for seven essential viral proteins [32]. which these (phosphoprotein, nucleocapsid protein, matrix protein, hemagglutinin-neuraminidase protein, RNA large protein, fusion protein, and V protein that is expressed by RNA editing of P mRNA) [33, 34]. As shown in Figure 1. Resistance to therapy is a significant obstacle in the treatment of cancer This study aimed to investigate and present the right techniques for propagating the oncolytic activity of the attenuated AMHA1 NDV Iraqi strain against Iraqi patient-derived glioblastoma multiforme cancer cells and to overcome treatment resistance.

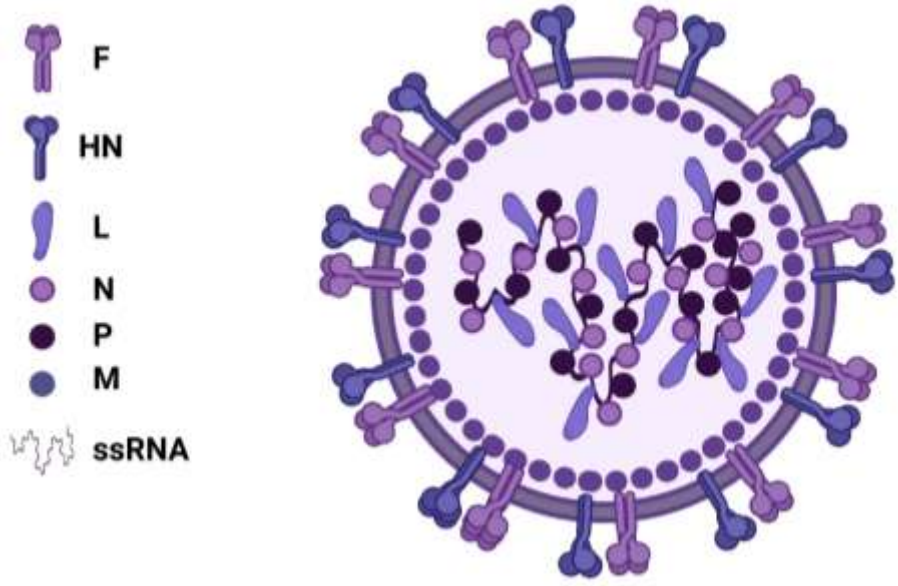

Figure 1: Schematic diagram of Newcastle disease virus structure. This figure was created using BioRender tools by the authors.

2. Material and Methods

2.1. Rosswell Park Memorial Institute (RPMI)-1640 Media

To prepare it, about 16.4 gm of RPMI-1640 powder was resolved in 800 mL of D.W., and the following components were added and mixed by a magnetic stirrer, 100 mL of fetal bovine serum (FBS), 0.5 mL of streptomycin (100 µg - ml^{-1}), 0.5 mL of Benzyl Penicillin solution (100 µg - ml^{-1}), 5 -10 mL Sodium bicarbonate (4.4%) to reach final pH 0f 7.6, 10.4 g L -glutamine, 10.4 g HEPES buffer. The pH was regulated to 7.2; the capacity was increased to 1 liter. Later, the solution was filtrated by a Millipore filter (0.22 µm) [35].

2.2. Minimum Essential Medium (MEM) Preparation

MEM was made in the following manner: In 600 ml, 11 grams of MEM powder (containing L-glutamine) was dissolved in triple-distilled water; after that, the other constituents were added: (2.2g) of Sodium bicarbonate powder, 10 mL of HEPES (Gibco, USA), (1 mL) 100 µg mL^{-1} of Ampicillin, 100 IUmL^{-1} of Streptomycin, and 100 mL of Fetal Calf Serum (FCS). After that, the medium was completed to one liter of triple distilled water; then the medium was sterilized by using the Nalgene filter (0.2 µm filter unit Nalgennunc, USA) [36].

2.3. Newcastle Disease Virus (NDV), Fertilized Eggs, and Chicken Red Blood Cells

Iraqi strain (AMHA1) of NDV was provided by the experimental Therapy Department /ICCMGR; the viral seeds were stored at -86 ℃ deep freeze. The embryonated eggs have been obtained from the Al-Khalil hatchery, located in the Abu Ghraib area, used in the cultivation of viral isolates (Six passages), used to isolate the virus from the pathological state as well as the cultivation of viral isolates, and the titration of viruses. RBCs of Chicken were harvested from the chicken brachial vein of the right wing in a syringe with heparinized and drawn about two milliliters, and to determine the titer of the virus was used blood [37].

2.4. Ahmed-Majeed-Glioblastoma-Multiforme-2005 (AMGM5) Cell Line

Dr. Ahmed Al-Shimmeri of ICCMGR generously donated the Ahmed-Majeed-Glioblastoma-Multiforme-2005 (AMGM5) cell line. A human brain GBM cell line was isolated from a 72-year-old Iraqi man who underwent surgery for an intracranial tumor. This cell line was cultured on RPMI-1640 for five days and a passage (35-40 of AMGM5) cell line was used in this investigation [15].

2.5. Vero Cell Line

Vero cells were produced from a healthy adult African green monkey's kidney in 1962. The cells were maintained in MEM with 5% FCS and passages (89-90) were employed in this study.

2.6. Methyl Thiazolyl Tetrazolium (MTT) Solution

A powder, of 0.5g of MTT is dissolved in 50mL of PBS to produce a concentration of 10 $mgmL^{-1}$; the generated solution is filtered through a 0.22 µm Millipore filter syringe. The product is then stored in sterilized-dark vials at -20 ^{0} C (freezer). The solution was used within up to six months of preparation [38].

2.7. Experimental Procedure

2.7.1. Virus Propagation

Experimental Therapy Department / Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) generously contributed frozen seeds of an Iraqi isolate of NDV. The antibiotics (both penicillin (1000 $IUmL^{-1}$) and streptomycin (10 $mgmL^{-1}$) were added to the viral sample after it had been thawed. The sample was centrifuged at 4℃ for 30 minutes at 3000 rpm. Initially, any large particle matter and trash would be removed. The supernatant was collected and kept at -86 ℃ in a tiny tube [39].

2.7.2. NDV Propagation in Chicken Eggs Embryonated

To guarantee the embryo's vitality, the eggs were first scanned using an egg candling lamp. All eggs with a dead embryo or that were non-fertile were discarded, and the air sac of the eggs and an appropriate injection site were marked off on the eggshell, taking into account that no major blood veins were present [40]. Before inoculation, each egg's surface was sterilized with 70% ethanol and iodine at the inoculation station, and then a sterile needle was used to inject 100 µL of the prepared virus into every egg's allantoic fluid, with the injection hole sealed with sterile wax. Embryo viability was checked daily after incubation in the incubator with humidity at 37℃. Embryos that had perished after 42 h. were isolated and re-incubated; embryos that had died were then transported to the refrigerator for 6h. Then, using sterile syringes, begin the harvesting process (collecting the allantoic fluid). Allantoic fluids that were unclear or bloody were discarded, and debris was removed by centrifuging at 3000 rpm for 10 min. at 4℃, taking the supernatant, sterilizing it using a 0.45 µm Nalgene filter unit, and finally putting it into Eppendorf tubes and storing it at -86℃. The virus then passages into embryonated eggs to obtain high titers, and the virus was measured in infected eggs until it reached 8 - 10 $HAUmL^{-1}$ [41].

2.7.3. Chicken RBC Solution Preparation

A healthy chicken's blood was collected into a heparin-treated tube to make the chicken RBC solution. Chicken blood (2 mL) was placed in a heparinized tube with seven mL of cold PBS, and after that put in the centrifuge at 4 ºC and 1000 rpm for 10 min. PPS was used to wash the blood 3 times. then removed the supernatant and RBCs pellets were collected. Then the (0.1ml) of RBCs pellets were added to PBS (10ml) to provide one percent of the RBC solution, which was used in the hemagglutination test [42].

2.7.4. Hemagglutination (HA) Test

According to the method described by Cunningham and his co-workers [40]. The H.A test was performed in the steps: The first step was to prepare the chicken red blood cells RBCs, which were taken from a bird in good health in a tube that had been heparinized, and it was washed with PBS three times by centrifuged at 3000 rpm at 4℃. Second, remove the supernatant and take 0.1 mL of cleaned RBCs, then dilute with PBS to 10 mL to make a one percent RBCs solution for this test. Third, 50 µL of cold, stirred PBS were dispensed into each well of a 96-well microliter plate with a plastic U-shape bottom. In the first well, 50 µL of infective allantoic fluid containing the virus was inserted, and a two-fold serial dilution was performed. The final step was to add 50 µL of 1% RBC solution to each well of the microplate, gently shake it, and leave it at room temperature for 15 minutes to observe and record the hemagglutination results. The lowest viral concentration apparent in observable chicken erythrocyte agglutination was described as an NDV Hemagglutination Unit (HAU).

2.7.5. Tissue Culture Infection Dose 50 ($TCID_{50}$) Test

TCID50 is the amount of pathogenic factor needed to infect 50 percent of inoculation cell cultures. To determine a 50 percent infection dosage in tissue cultures, NDV was titrated on the Vero cell line in a microtiter plate. Vero

cells were seeded at 7×10^3 cells/well (in 96-well microtiter plates with a flat bottom). After 24 hr. The viral solution was serially ten-fold diluted when the growing medium was discarded. In 8 wells (50 µL per well), the cells were injected. From each dilution, the control cells were injected with serum-free media (SFM), as shown in Figure 2. The plate was incubated for 2h. after being coated with a sterile adhesive cover, at room temperature to facilitate viral attachment and adsorption Then, incubated at 37 °C and every day for (three-five days) was checked the infected plate [43]. On the Vero cell, the NDV titer was calculated as described by Skalka *et al*., [44]. After that, the medium was removed, and the wells were filled with 50 µL of crystal violet stain for 30 minutes at 37°C, the dye was removed via aspiration. The viral dilution that caused a 50% cytopathic effect (CPE) was used to calculate the titer by the equation:

$$\mathbf{log10\ (TCID_{50}/ml) = L + d(s\text{-}0.5) + log\ (1/v)} \quad (1)$$

Where: L = negative log10 of the most concentrated virus dilution tested in which all wells are positive, d = log10 of dilution factor, s = sum of individual proportions Pi, Pi = an account proportion of an individual dilution, (Amount of positive wells/total amount of wells per dilution), and v = volume of inoculum ($mLwell^{-1}$), The virus titer could also be calculated using the method reported by Reed and Muench in 1938 [45].

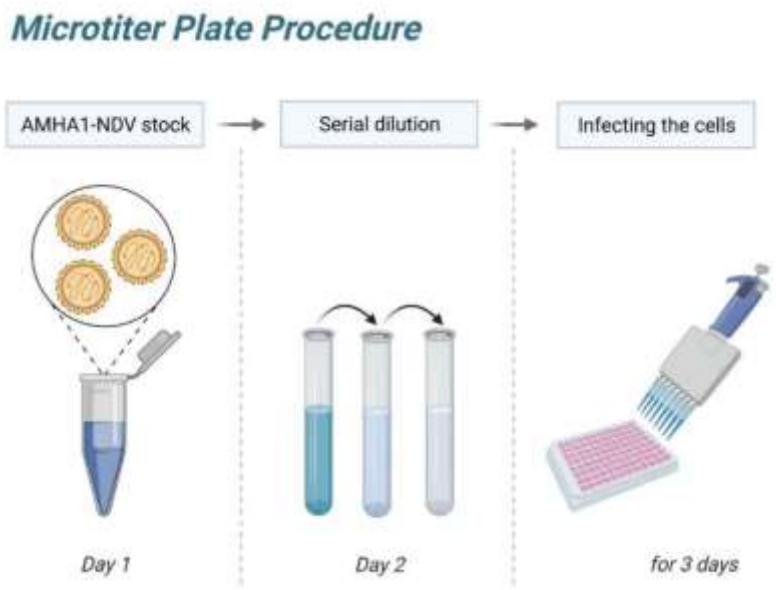

Figure 2: Serial fold dilution in TCID50 test. The figure was created using the Biorender tools.

2.7.6. Cytotoxicity determines by using an MTT Assay

To generate monolayer cells, AMGM5 cells were seeded at 1×10^4 cells/mL in 96 well microplates in RPIM growth media and incubated at 37ºC overnight. NDV were then incubated for 72 hours after being exposed in triplicate at varied multiplicities of infection (MOI: 0.1,0.3,0.5,1,3,5,10, and 20). After that, a 50 µl concentration of MTT viability stain was applied. Dimethyl Sulfoxide (DMSO) 150 µl was added to each well after 3 hours of incubation at 37°C. A micro-ELISA reader was used to measure the absorbance at 570 nm. The findings of the experiment were expressed as a percentage compared to the control cells' growth [46, 47].

2.7.8. Statistical Analysis

The one-way analysis of variance (ANOVA, Tukey Test) was used to analyze the significance of variances across groups, with statistically significant differences defined at * p 0.05 or ** p 0.01. The statistical significances were calculated using Graph Pad Prism version 6 and the data were displayed as mean standard deviation (Graph Pad Software Inc., La Jolla, CA).

3. Results and Discussion

3.1. NDV Propagation in Chicken Eggs Embryonated

The findings revealed that the NDV strain from Iraq can destroy chicken embryos between 24 -72hr. following inoculation of chicken embryonated eggs. As a result, the strains were labeled as velogenic [48]. The embryonic death that occurred within 24 hours of inoculation was deemed nonspecific, and the eggs were discarded [49]. Figure 3A & B shows bleeding in infected embryos compared to uninfected embryos in the control group.

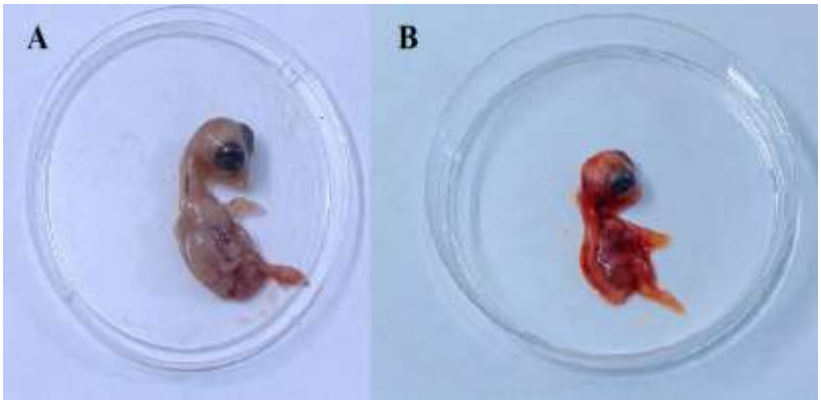

Figure 3: (A) control not infected showing no alterations compared to **(B)** Skin hemorrhage in chicken Embryos after inoculation with NDV.

This outcome is consistent with what other researchers have found Alexander *et al.,* [50]. The virulent local NDV is also known as a virulent viscerotropic strain, as demonstrated by Zahid and Bunny [51]. The finding of this study is also compatible with Al-Shammari *et al.,* who detected the virus killed embryos in less than 72 hr. with extreme hemorrhage in infected embryos [24].

3.2. Haemagglutination Test (HA)

After virus purification using centrifugation and a 0.45 µm Nalgene filter, virus quantification was performed using a HA test, following viral propagation and purification, which revealed a positive outcome as a model haemagglutination lattice pattern of chicken RBCs 2^7 or 128 HAU as seen in Figure 4.

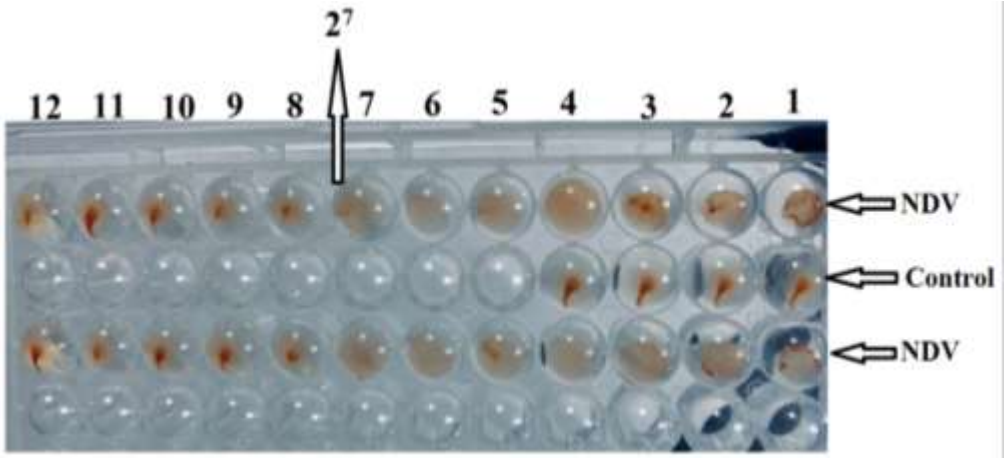

Figure 4: The HA test displays both agglutinated and non-agglutinated RBCs in a part of the 96-well plate. It's worth noting that the reading was performed twice. Erythrocyte agglutination results at the bottom of a distinct lattice, whereas negatory hemagglutination is distinguished with an acute redpoint.

It is worth noting that the pellet and retinal capillary morphological features were both visible to the naked eye. Agglutination began on the first well plate and continued until the seventh well plate, while the remaining plates had no trellis form of RBCs, but a red button appearance of RBCs at the bottom of the plates, indicating that no

agglutination of RBCs had occurred in those wells. This was because non-agglutinated RBCs will drop to the bottom of the well plate and form an RBC pellet, whereas agglutinated RBCs will create a lattice or trellis structure [52]. By determining the HN gene of the virus, the HA test has been used to assess the NDV activity. The NDV forms a mesh by adhering to red blood cells via certain receptors, and this gene product is involved in protein-protein interaction [53].

3.3. Infectious Dosage of Tissue Culture 50

TCI_{50} is the infectious titer of any virus capable of inducing cytopathic effects (CPE) in tissue culture for 3 to 20 days while the cells in the culture remain alive and are assessed using TCI50 tests. His method is used to calculate the number of infectious viruses in a sample. Not all viral types cause CPE in tissue culture, and to notice a CPE, the cell line and virus must be exactly matched [54]. To examine the molecular mechanisms underlying viral pathogenesis, NDV has been adapted for the Vero cell line in several prior investigations [55]. The earliest signs of CPE were a large number of restricted cells with granulation and shrinking of cytoplasm, which finally led to the infected cells being separated and floating in the culture fluid after 24 to 48 hrs. After 72 hrs., cell rounding, aggregation, and syncytia development were seen, followed by the detection of infected cells with the formation of huge round empty spaces. Within the same period as the analysis of infected cells, the same observations were not observed in the control cell culture, as shown in Figure 5. Infectious dosage of tissue culture Because $TCID_{50}$ analysis permitted defining and standardizing the number of virus particles given per cell, $TCID_{50}$ experiments were performed to estimate the multiplicity of infection (MOI) of the virus that was driven by small total particle yield, which indicates viral particles per cell [56]. The $TCID_{50}$ was calculated by measuring the NDV titter in Vero cells based on the link between viral dilution and syncytia formation in seeded wells with Vero cells and by using the equation provided by [36]. $TCID_{50}$ was 2×10^6 viral unit/ mL.

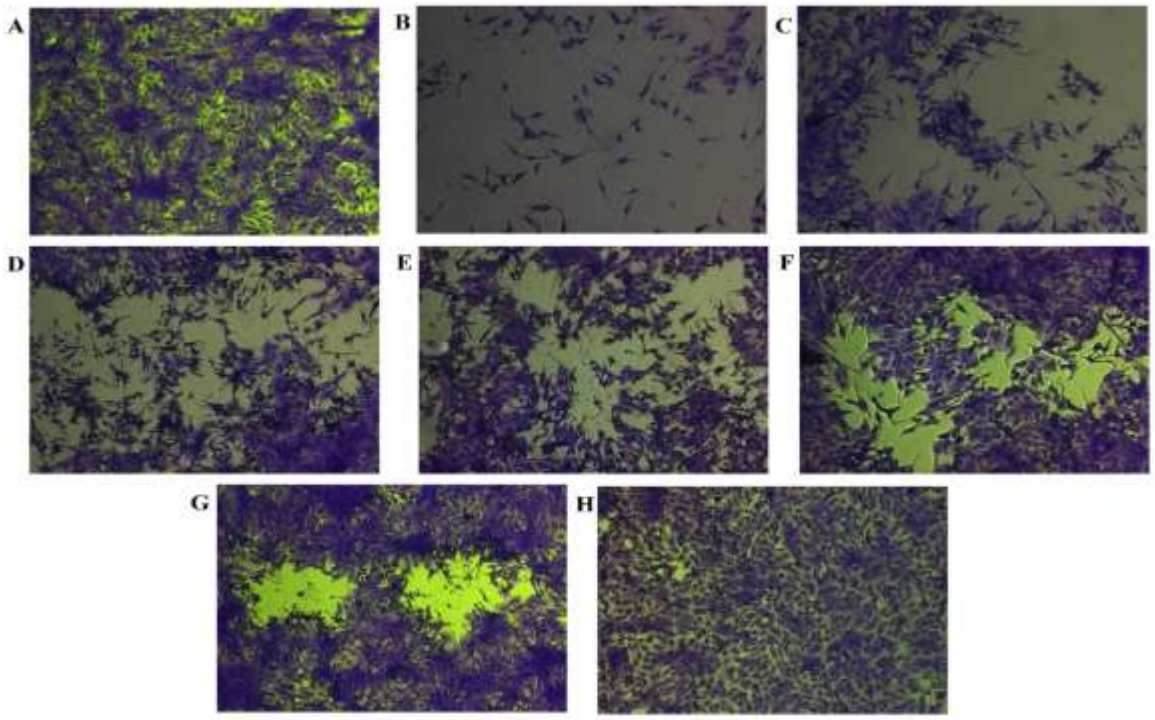

Figure 5: The cytopathic effect of the NDV on Vero cell lines by utilizing the virus titration ($TCID_{50}$). (A) Control not infected cells, (B) infected with the first dilution of NDV; (C) infected with the second dilution of NDV; (D) infected with the third dilution of NDV; (E) infected with the fourth dilution of NDV; (F) infected with the fifth dilution of NDV; (G) infected with the sixth dilution of NDV; and (H) infected with the seventh dilution of NDV.

3.4. Cytotoxic Effect of NDV on AMGM5 Cells

Figure 6 shows the effect of NDV on growth inhibition G.I percent of the (AMGM5) cancer cell line after 72 hours. The results of G.I percent for the AMGM5 cancer cell line after 72 hours at MOI (20) viral particles revealed higher significant inhibition (88.5%) in comparison to the eighth MOI (0.1) viral particles which showed a lower inhibition rate (48.5%) with highly significant variation between the MOI. And as shown in Figure 7, the cytotoxicity effect of the NDV on AMGM5 cell lines by utilizing the eight MOI of the virus.

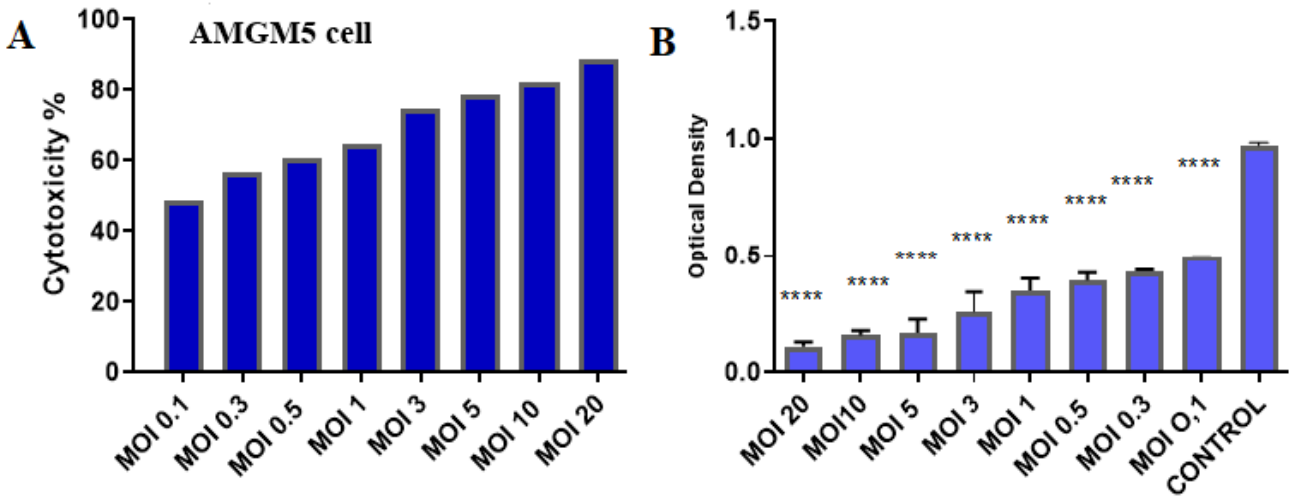

Figure 6: **(A)**The cytotoxic effect, **(B)** Optical density of NDV on AMGM5 cell lines were studied using different MOI (0.1,0.3,0.5,1,3,5,10, and 20) for 72 hours. The MTT viability assay was used to measure the cytotoxicity of the NDV, it was shown in terms of the rate of G.I. for Cancerous cells, and all MOI demonstrated significant inhibition.

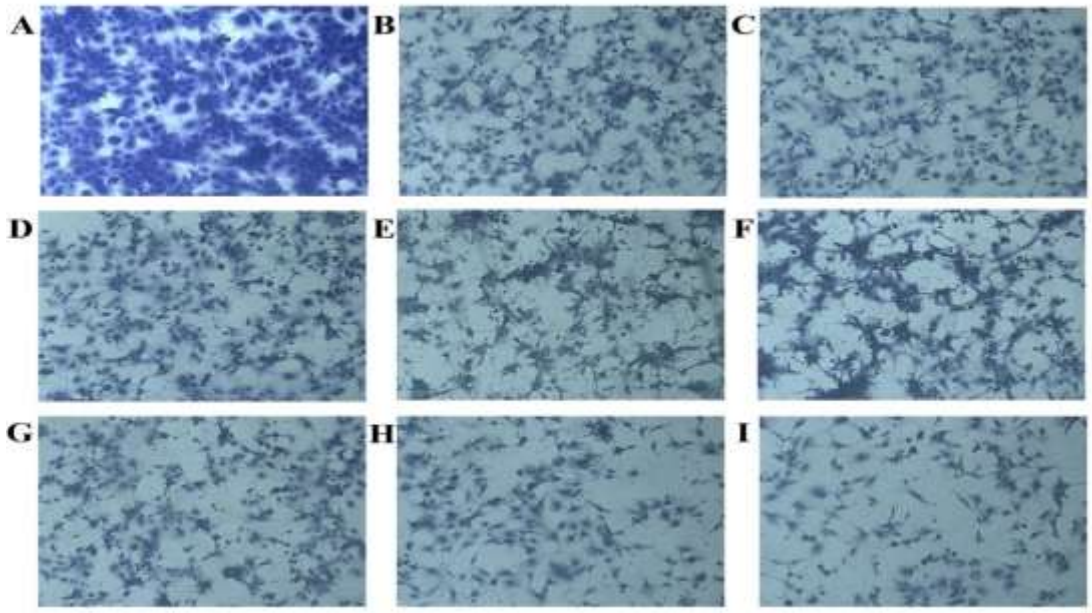

Figure 7: The cytotoxicity effect of the NDV on AMGM5 cell lines by utilizing the eight MOI of the virus. **(A)** Control not infected cells, **(B)** infected with the 0.1 MOI of NDV; **(C)** infected with the 0.3 MOI of NDV; **(D)** infected with the 0.5 MOI of NDV; **(E)** infected with the 1 MOI of NDV; **(F)** infected with the 3 MOI of NDV; **(G)** infected with the 5 MOI of NDV; **(H)** infected with the 10 MOI of NDV; and **(I)** infected with the 20 MOI of NDV.

All eight effective NDV doses (viral particles) had a substantial cytotoxic effect ($p<0.05$) after 72 hours, according to the results. With the increase in MOI, the G.I percentage increased (viral particles). These findings suggest that NDV requires more numbers to enter a cell and interfere with cell receptors, cytoplasm, or nuclear membrane, causing cancer cells to kill. It's worth noting that oncolytic paramyxoviruses are capable of killing a wide range of human cancer cells. like Measles virus (MV), (NDV), Sendai virus (SeV), and Mumps virus (MuV) [57]. The intriguing trait is the oncolytic ability of viruses, which can infect and proliferate in cancer cells while selectively killing cancer cells while leaving normal cells unharmed [58]. Figure 8 shows the AMHA1-NDV oncolytic mechanism.

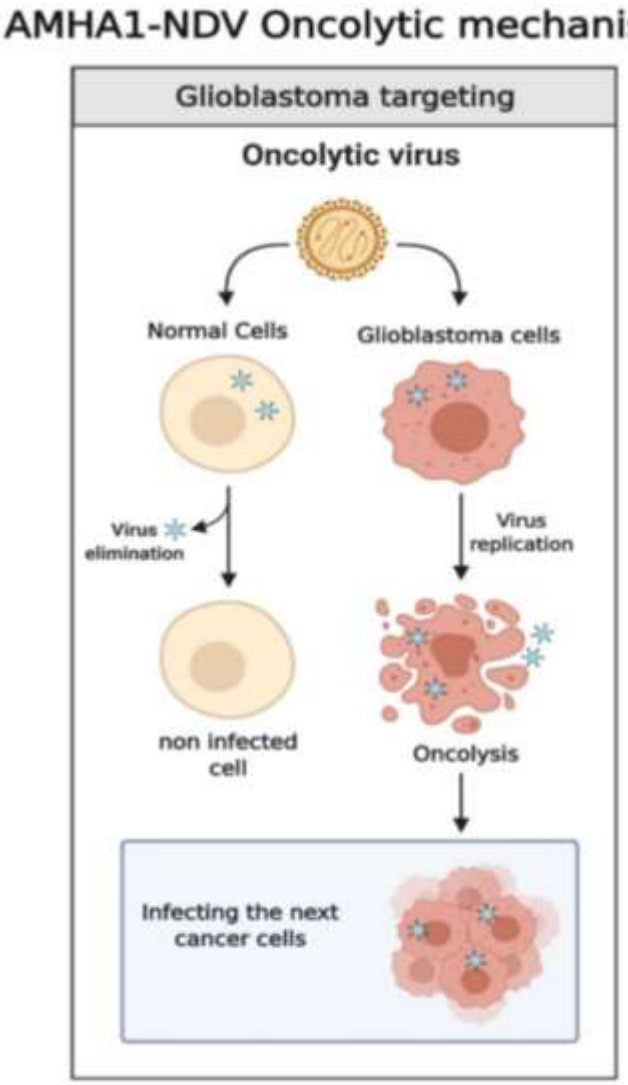

Figure 8: The proposed mechanism for NDV-AMHA1 strain against Iraqi patient-derived glioblastoma multiforme.

The tumor cells are a preferred substrate for oncolytic viruses particularly the paramyxoviruses for many interrelated reasons, including the cancer cells' high metabolic rate.; thus, they can sustain virus propagation even if their host-modifying functions are defective. Normal cells begin p53-dependent suicide programs in response to viral infection, which kill aberrant (infected) cells before they start shedding viral offspring [59-60]. Because functioning p53 is frequently lost in malignancies through numerous pathways, malignant cells do not normally commit suicide in response to viral infection and efficiently complete virus production [61, 62]. As cell surface receptors, NDV uses sialic acid-containing sialoglycoproteins. The abundance of sialoglycoproteins on

the surface of cancer cells increases the virus's preference for malignant cells over normal cells, resulting in a selective cytolytic effect in primary tumors and metastases. Hemagglutinin-neuraminidase (H.N.), one of the glycoproteins found in the envelope of NDV, is crucial for recognizing and binding sialic acid-containing receptors to cell surfaces [63-65]. By intratumoral injection, the Iraqi NDV strain demonstrated a potent immune-stimulatory effect because it motivates IL-2 and IFN-gamma and draws CD56 natural killer cells and CD8 cytotoxic T lymphocytes into tumor tissue that has been infected [66].

4. Conclusions

The AMHA1-NDV attenuated Iraqi strain can infect, replicate and cause cytotoxicity and cell death in glioblastoma multiforme cancer cells derived from an Iraqi patient through cytolysis. Vero cells can be utilized as permissive cells for NDV since NDV can cause a cytopathic impact in these cells. For future suggestions to bioconjugation of purified NDV with chemotherapy to improve Glioblastoma death and overcome resistance to chemotherapy when used alone, or with nanoparticles and study how this combination could induce immune system. Or study the synergistic effect of NDV with anther therapies as combination therapy and its effects on apoptotic induction in tumor cells (*in vivo*). These promising results encourage more studies to be translated into clinical trials to save cancer patients from this untreatable disease.

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Conflict of Interest

There are no conflicts of interest declared by the authors.

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