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Cytotoxicity of Newcastle Disease Virus (NDV) against cultured breast cancer cell lines

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Abstract: The second leading cause of death for women is breast cancer. The disease has been successfully managed by conventional therapies. The aim of study to found novel strategies, such as oncolytic virotherapy, were required to fight it. x given by ICCMGR, was cultivated using chicken eggs from commercial hatcheries in Al-Hur area, a suburb of Holy Kerbala province. Propagated NDV induced considerable hemorrhaging in infected embryos compared to unaffected control embryos and destroyed all embryos within 48 hours. For additional testing, allantoic fluid containing the virus was collected, purified, and kept at -20°C. Hemagglutination (HA) assay was employed to quantify the NDV titer which show positive findings as a typical haemagglutination mesh pattern of chicken red blood cells at 2^8, or 256 HAU. Using Vero cells and Flint's algorithm to calculate the Tissue Culture Infection Dose 50 (TCID50) test, the viral dilution and syncytia growth in the seeded wells were linked, and the result was found to be $3.5 * 10^6$ viral unit/ml. The multiplicity of the viral infection (MOI) 1,2 and 3 was prepared and tested on Amj-13 and MCF-7 of which cell viability and growth inhibition rate using MTT assay was examined. Results of Optical Density (OD) and Inhibition Rate Growth Percentage (IR%) were calculated using Statistical Analysis System (SAS) to determine Least Square Means (LSM) to compare the significance difference between the mean values at $p \leq 0.05$. There were proportional significant increases, ($P \leq 0.05$), in inhibitory rate of both cancer cell lines treated with all concentrations of NDV alone compared with control of which the highest inhibitory rate was recorded at concentration (3 MOI) suggesting the more viral particles, the greatest cytotoxicity caused that reached 73.14% and 67.7 % respectively. The cytopathic effects (CPE) caused to cell lines due to the exposure NDV was observed under inverted microscope. Cells suffer morphological changes including granulation and shrinkage of cytoplasm, which lead eventually to separation and floating of the infected cells in the culture media, after 72 hrs. large round empty spaces were so apparent. These observations were not detected in control cell culture within the same time of examination of infected cells. The results were concluded promising results in not just eradicating cancerous cells, but as potential cancer vaccine.

Keywords: NDV, CPE, , Vero cell line, MCF-7, Amj-13.

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Introduction

Breast cancer is ranked as the most common in years between 1986-2011 according to the Iraqi Cancer Registry (1). As a result of their ability to selectively infect cancerous cells while inflicting minimal harm to untransformed or normal tissues, oncolytic viruses have become one of the most popular forms of treatment (1).

Natural strains or those created through genetic engineering Oncolytic viruses are a diverse group of DNA and RNA viruses that can be used as in situ vaccinations, added to other immunotherapies, or equipped with immunomodulatory transgenes (2). Since the Newcastle disease virus (NDV) appears to preferentially infect and destroy malignant cells, it has been widely employed in the past 20 years to treat cancer in people (3). This virus can be used to cure cancer because it is not harmful to mammals and only reproduces in cancer cell, In the U87MG cell line, for instance, virulent NDV strain, V4UPM, was tested both in vitro and in vivo and shown antiglioma effectiveness by taking advantage of tumor-specific defects in the interferon (IFN)-mediated antiviral response of cancer cells (4).

The other recognized selectivity mechanisms were deficiencies in antiviral signaling pathway activation as well as deficiencies in both intrinsic and extrinsic apoptotic pathways (5).

The direct impacts of this virus on living things were studied in several early studies, as well as the mechanisms by which NDV kills tumors, including necrosis due to excessive replication, generation of tumor-specific immunological memory leading to immunogenic cell death in a glioma mouse model (6).

Recombinant Newcastle disease virus (rNDV) has shown an anticancer effect in preclinical studies (7).

The viral surface HN protein has been shown to be an important part of this virus' immunostimulatory properties. Around ten hours later, The surface of tumor cells are altered by NDV infection (increased expression of the viral HN and F proteins). Al-shamery (2003) discovered the Iraqi strain of Newcastle disease virus has both in vitro and in vivo oncolytic activities in many cell lines. Immuno-compromised animals with murine mammary adenocarcinoma and mice with human stomach cancer (fibrosarcoma) were treated with this novel drug. In experimental animals, the Newcastle disease virus (NDV) demonstrated a significant decrease in the occurrence of cancer and was shown to be safe (7). Since then, a number of studies have been conducted in which NDV has proven to have numerous benefits and great promise for the treatment of cancer. Such advantages include NDV's capacity to introduce its HN protein into the surface of many tumor cells to promote proliferation.its capacity to damage DNA, resulting in apoptosis and cell death. Both intrinsically and extrinsically (8). Additionally, it has been revealed that the Iraqi NDV activates the endoplasmic reticulum stress route of death (9). More notably, NDV replication in tumor cells has shown a preventative method for cancer cells' production of chemicals that promote angiogenesis (10).

Material and methods

Propagation of Newcastle disease virus

Commercial hatcheries in Al -Hur region, suburb of Holy Kerbala province provided Seven days old of incubated eggs which kept at 37 °C and in a humidified shaker incubator. The eggs were cleaned with iodine and sprayed with 70 percent ethanol before being checked for the survival of the embryo in which dead ones were discarded. Embryonic eggs were candled, and the air space was identified when they were 9 days old. Then, (0.1ml) of virus was introduced into the allantoic fluid of the eggs was obtained

from the Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetics Research in AL Mustansiriyah University (ICCMGR) using a tiny sterile needle. Melted candle wax was used to seal the egg's puncture hole (11). The eggs were checked each day for signs of death, such as blood vessels that had burst or embryos that remained immobile after 24 hours. The eggs were checked each day for signs of death, such as blood vessels that had burst or embryos that remained immobile after 24 hours. To prevent blood contamination when harvesting the allantoic fluid, the remaining eggs were retained until 90 percent of the embryos had died. The embryo was then immediately put in the refrigerator at 4°C after passing away.

After 12 to 24 hours, the allantoic fluid was removed using a sterile syringe (the clear allantoic fluids were harvested in 50 µL sterile containers). To cleanse them, cold centrifugation (3000 rpm, 30 minutes, 4°C) was employed. After being filtered via Nalgene syringe filters with a particle size of 0.4-0.22 µL and then transferred to Eppendorf tubes, the allantoic fluid was stored at -20°C.

Hemagglutination (HA) assay

The test was developed to quantify the NDV titer required for inhibition testing using the virus's capacity to agglutinate blood. Blood was drawn from a healthy chicken's bronchial vein beneath the wing and placed in a heparinized tube (with PBS and EDTA composition to prevent the blood from clotting). The blood was centrifuged in PBS at a speed of 1000 rpm. Next, the pellets and supernatant were redissolved in PBS and centrifuged three times to maintain a 5% RBC solution. We employed a U-shaped 96-well microtiter plate with 50 µL of PBS (SPL, Korea) in each well. The first well received 50 µL of allantoic fluids before experiencing a two-fold serial dilution followed by 50 µL of the 5% RBC solution. After giving the plate a little shake, the results were recorded while the hemagglutination developed and the plate was left at room temperature. The Newcastle disease virus was quantified as one hemagglutination unit (HAU), the smallest viral concentration inducing clearly agglutinated chicken erythrocytes (12).

Tissue culture laboratory work

Maintenance of cell lines

Cell lines provided by ICCMGR reached monolayers of confluence, they were sub cultured. The cell line sheet was washed with 2ml of the trypsin-versene solution after the growth medium was decanted off. After adding two to three ml of trypsin-versene, the flask gently shook. The flask was then decanted one more to get a little amount of trypsin-versene solution covering the cell surface (approximately one milliliter). The cells were then incubated at 37°C until they separated from the flask. Cells were initially dispersed in growth media to the required concentration before being redistributed into culture flasks and re-incubated at 37°C.

Tissue Culture Infection Dose 50(TCID50) test

According to Pedersen, Vero cells were seeded at 7000 cells/well in 96-well microtiter plates with a flat bottom (13) After 24 hours, the growing medium was removed, and the viral suspension was serially diluted tenfold. 0.9 mL of the diluent was added to all the following eppendorf tubes. The original undiluted viral sample was pipetted into the first eppendorf tube after vortexing. 0.1 ml of the first tube's

contents were added to the second tube, and so on for the remaining tubes. The cells were divided into 6 wells for each dilution (50 l per well), and the control cells received injections of serum-free medium (SFM). The plate was covered with a sterile adhesive sheet and incubated at 37 °C for 2 hours at room temperature for (three-five) days, the infected plate was inspected every day. The cells were cleaned, fixed, and stained to detect the presence of viruses. Each well was looked at under a microscope to see if it contained one or more infected cells, or whether it did not (14). According to Flint, the NDV titer was assessed on Vero cells (15). In this case, the titer was determined as the viral dilution that generated a 50% cytopathic impact (CPE): log10 (TCID50/ml) = L + d(s-0.5) + log (1/v)
Where:
L = the most concentrated virus dilution tested, with a negative log10 of all wells being 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).
v = volume of inoculum (ml/well) also the titer can be calculated as described by Reed and Muench (1938).

Cytotoxicity assay of NDV on different cancer cell lines.

In sterile tubes, dilutions of NDV at (1, 2 and 3) MOI were created using cold serum-free media. In 96 well plates, the AMJ13 and MCF7 cell lines were planted at 10,000 cells per well and incubated overnight at 37°C. The medium was removed from the microtitration plate and the cells were infected with NDV. Then, for 2 hours at room temperature, 300 µl of each NDV dilution was applied to each well, allowing virus attachment and penetration. A micropipette was used to extract virus solution from each well. Virus solution was withdrawn as quickly as possible from each well using a micropipette, and 200 µl of warm serum-free medium was added. The plate was then The AMJ13 and MCF7 cell lines were seeded at 10,000 cells per well in 96-well plates and incubated at 37 C overnight. The microtitration plate's media was drained, and NDV was then introduced to the cells. 300 µL of each NDV dilution was then added to each well, enabling the virus to adhere and penetrate, for 2 hours at room temperature. In addition, negative controls, were included for each experiment to ensure validity of the assay. The plates returned to the incubator at 37°C, the times of exposure were (72 hrs.).

MTT Assay

MTT assay is a rapid colorimetricsemi-automated enzyme based assay (16).

Using an MTT test kit (Elabscience®, USA), cell growth and cytotoxicity were quantified. Medium was removed and 50 µL of 1 × MTT working solution was added to each well of which incubate at 37°C, 5%CO2 Cell incubator for 4 hrs. 150 µl of DMSO (dimethyl sulphoxide) (Santa Cruz Biotechnology, USA) was added causing the crystals that were still in the wells to be solubilized. The mixture was then incubated for 15 min while being shaken (16). The absorbency was measured at a wavelength of 492 nm using a microplate reader (Expert Plus Reader, Asyshitech, Austria). At least four times were run through the test in triplicate. SEM was used to compute the mean viability of the treated cells for each dilution as a percentage in

comparison to the control wells treated with medium alone (100% survival).

Finally, the percentage of cytotoxicity was estimated based on the inhibiting rate of cell growth according to, (17) as below:

Inhibition rate = (Mean of control – mean of treatment/ Mean of control) × 100%

Staining of the cells

Cell viability was evaluated following the exposure period by removing the medium from the plate and adding 50 µL of Crystal violate. then let to rest for 20 minutes at room temperature. Before use, the plate was lightly tapped to remove any remaining liquid after being washed. This was done for at least two hours at room temperature.200 µl of methanol was added to each well and the plate was incubated at room temperature for 20 minutes with the cover on. A plate reader was used to measure the optical density at 570 nm (OD570) (18). Then, cells were carefully inspected using an inverted microscope (Olympus, Japan) at 10x, 20x, and 40x magnification.

Statistical analysis

Completely Randomized Design was used to process the data using one-way classification (Analysis of Variance, ANOVA) (CRD). Tables with mean values and standard error of the mean show the findings from all measurements. The Statistical Analysis System was used in the current study to determine the effects of various factors. (Stat Soft Inc., Tulsa, OK, USA) - SAS (21) Program and General Linear Model - GLM procedure were used (19). Least Square Means (LSM) were used, to compare the significance difference between the mean values at $p \leq 0.05$.

Results and discussions

Embryonated Hens eggs

According to the findings, isolated NDV kills all embryos before 48 hours and causes noticeable hemorrhaging in infected embryos compared to unaffected control embryos. as showing in figure (1). This demonstrates that the virus can cause mortality in a manner similar to previous velogenic strains (20). This study's findings concur with those of Al-Shammari et al. (8) who discovered that the virus destroyed embryos in less than 72 hours and caused severe hemorrhaging in infected embryos (8).

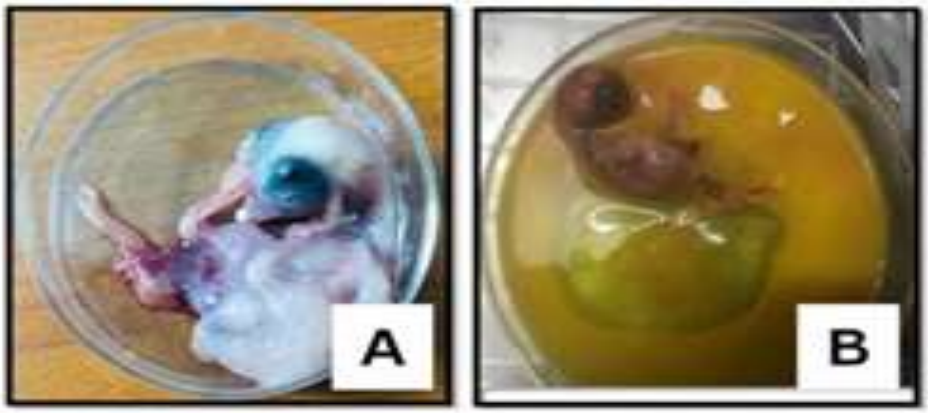

Figure (1): (A) Control not infected exhibiting no changes compared to (B) After NDV inoculation, skin haemorrhage occurs in chicken embryos.

Haemagglutination test

Haemagglutination tests that show positive findings as a typical haemagglutination mesh pattern of chicken red blood cells at 2^8, or 256 HAU, were used to quantify the virus.as shown in figure (2). The retinal capillaries and pellet were visible with the unaided eye. Agglutination began on the first well plate and continued until the eighth well plate; the remaining well plates do not have any RBCs in the trellis form.

Figure (2): Part of the 96 well plates containing both the agglutinated and non-agglutinated RBCs. It showed the agglutination of erythrocytes forms a distinctive mesh at the bottom, the negative hemagglutination is characterized by sharp red dot.

Tissue Culture Infective dose 50 (TCID50) of NDV.

According to recent findings, the initial sign of CPE was a significant number of confined cells with granulation and shrinking of cytoplasm within 24 to 48 hours. After 72 hours, the infected cells separated and floated in the culture medium. Cell rounding, aggregation, and syncytia formation were seen, and then infected cells were found with the development of large round empty spaces. Within the same time frame as the study of the infected cells, same observations were not seen in the control cell culture. Figure (3: A - E) using an inverted light microscope, display the cytopathic effects of the NDV on Vero cells that were previously discovered. wherein the cell rounded, the cytoplasm separated from the nucleus, and the nucleus condensed.

Nonetheless, there was no agglutination of RBCs in those wells, as evidenced by the red button appearance of the RBCs at the base of the plates. Nonetheless, there was no agglutination of RBCs in those wells, as evidenced by the red button appearance of the RBCs at the base of the plates (21).

Using an inverted light microscope, display the cytopathic effects of the NDV on Vero cells that were previously discovered. wherein the cell rounded, the cytoplasm separated from the nucleus, and condensed. On Vero cells, TCID50 of the Iraqi NDV strain was thrived. This was done to measure the multiplicity of the viral infection (MOI) while cells in culture are still alive and the infectious titre of the virus, which can produce cytopathic effects (CPE) over a period of 3 to 5 days. Using Flint's equation and the association between viral dilution and syncytia development in the seeded wells with Vero cells. TCID50 was $3.5 * 10^6$ viral unit/ml (15).

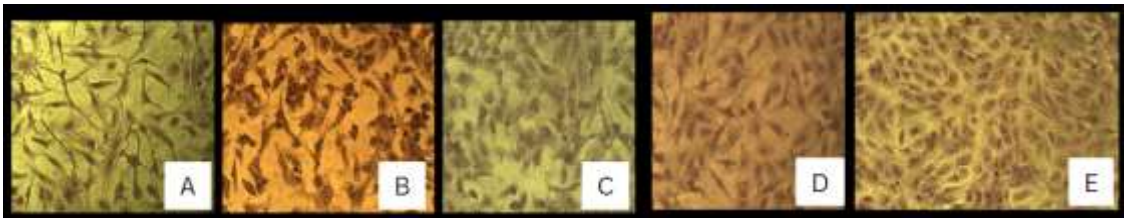

Figure (3: A - E): The cytopathic effect of the NDV on Vero cells used for virus titration (TCID50). (A) infected with first dilution of NDV; (B) infected with second dilution of NDV; (C) infected with third dilution of NDV; (D) infected with forth dilution of NDV; (E) Control (not infected). A cytopathic effect was clearly observed 72 hours after exposure, 10 X.

Cytotoxicity of NDV on AMJ13 cell line

NDV was cultured for 72 hours on AMJ-13 cells, and as the virus concentration (MOI) increased, the Cell Viability of cells steadily decreased, reaching its lowest value of 0.217 at that time (3MOI). As the viable cell ran out of virus at 1MOI, it improved and reached its maximum. ANOVA with the Least Significant Difference (LSD) test was used to compare between means. In all concentrations of NDV, there was a substantial (P 0.05) decrease in cell growth, as shown in table (1).

Table (1): Cell viability after exposure to NDV alone on AMJ13 cell line. O.D: optical density; MOI: multiplicity of infection.

Group	OD Mean ± SE
MOI 1	0.352 ±0.03 b
MOI 2	0.289 ±0.02 b
MOI 3	0.217 ±0.02 b
Control	0.809 ±0.05 a
LSD value	0.307 *
P-value	0.0271
Means having with the different letters in same column differed significantly. * (P≤0.05).	

Inhibition Rate percentage (I.R) %

There were significant increases, (P ≤ 0.05), in inhibitory rate of AMJ13 cancer cell line treated with all concentrations of NDV alone compared.

Cytopathic Effect (CPE)

Amj13 cell lines in figure (5) suffered prior to exposure cytopathic effect represented in rounding of the cell,

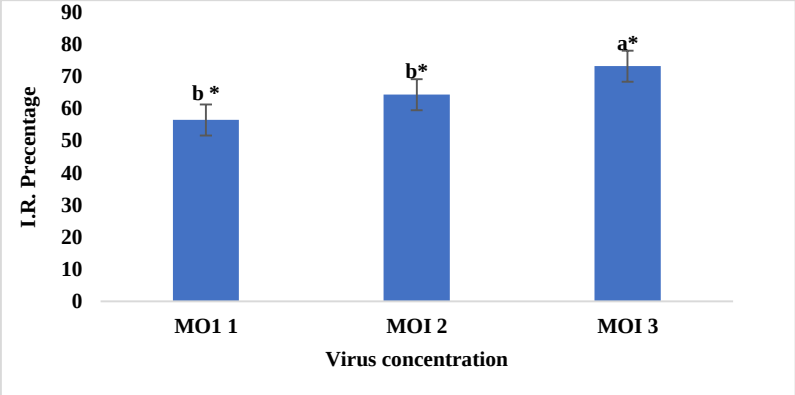

Figure (4): Inhibitory rate of NDV alone on AMJ13 cell line. Different letters: significant at (P ≤ 0.05). *: significant compared with control at levels (P ≤ 0.05). % I.R: inhibitory rate.

Cytotoxicity of NDV on MCF-7 Cell Line

NDV was incubated for 72hrs. on AMCF-7 cells, the O.D. measurement was gradually decreasing with the with control. The highest inhibitory rate at concentration (3 MOI) was (73.14 %), (Figure 4).

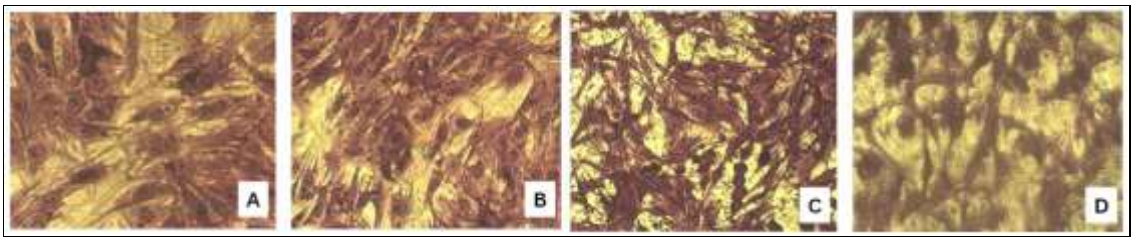

Figure (5): Cytopathic effect of NDV on AMJ-13 cell line: (A) control. (B) NDV of 1 MOI. (C) NDV of 2 MOI. (D) NDV of 3 MOI.

Table (2): Cell viability after exposure to different concentrations of the NDV on MCF-7 cell line.

Group	OD Mean ± SE
MOI 1	0.362 ±0.02 b
MOI 2	0.300 ±0.02 b
MOI 3	0.243 ±0.02 b
Control	0.752 ±0.05 a
LSD value	0.289 *
P-value	0.0392
Means having with the different letters in same column differed significantly. * (P≤0.05).	

Cytoplasm separation from nucleus and condensation of nucleus were observed. Increase in the virus concentration (MOI) recording the lowest of 0.243 at (3MOI). LSD value was 0.289 with significant p value of ≤0.05 in all virus MOI concentrations confirming the powerful killing potential of the virus on MCF-7 cell line.

Inhibition Rate Percentage (I.R.) %

There were significant increases, ($P \leq 0.05$), in inhibitory rate of normal MCF-7 cancer cell line treated with all concentrations of NDV alone compared with control. The highest inhibitory rate was 67.7 % at 3 MOI, respectively, (Figure 6).

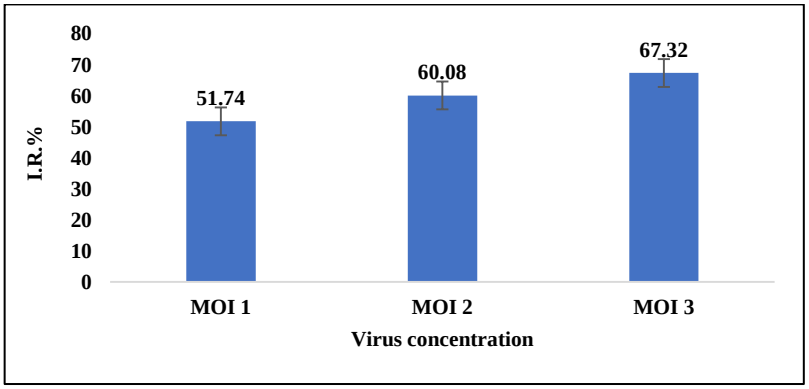

Figure (6): Inhibitory rate of NDV alone on MCF-7 cell line. Different letters: significant at ($P \leq 0.05$). *: significant compared with control at levels ($P \leq 0.05$). % I.R: inhibitory rate.

The lower inhibition rate was nearly 52 %. The reductions, ($P \leq 0.001$), in cell proliferation were observed in the presence of all concentrations at 72 hr. after exposure.

Cytopathic Effect CPE of NDV

The cytopathic effects (CPE) caused to MCF-7 cells due to the exposure NDV appeared in figure (7) to that cells suffer morphological changes including granulation and shrinkage of cytoplasm, which lead eventually to separation and floating of the infected cells in the culture media, after 72 hrs, large round empty spaces were so apparent. These observations were not detected in control cell culture within the same time of examination of infected cells. MCF-7 cells normally have an epithelial-like shape and monolayers form dome structures due to fluid accumulation between the culture dish and cell monolayer.

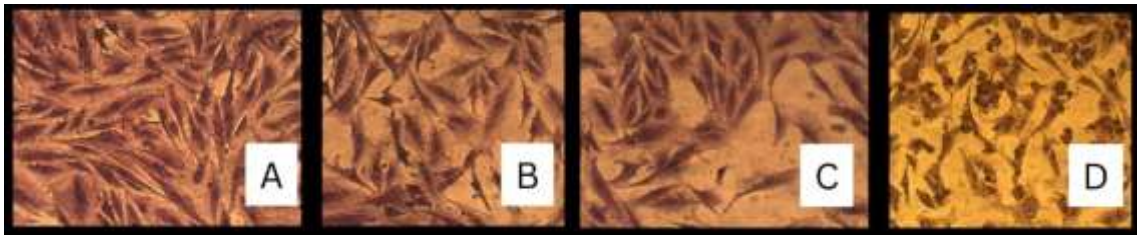

Figure (7): Cytopathic effect of NDV on MCF-7 cell line: (a) control. (b) NDV of 1 MOI. (c) NDV of 2 MOI. (d) NDV of 3 MOI.

Conclusion

These observations were not detected in control cell culture within the same time of examination of infected cells. The results were concluded promising results in not just eradicating cancerous cells, but as potential cancer vaccine.

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