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# Oncolytic effect of Newcastle disease virus (NDV) strain AF2240 and V4-UPM on hypoxic and normoxic osteosarcoma cells

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## ABSTRACT

**Aims:** Hypoxia is believed to be one of the key components contributing to the clinical resistance of cancer therapies. Alternative strategies are under investigation to overcome this resistance and the oncolytic virus stands amongst the others. Newcastle disease virus (NDV) has been demonstrated to possess oncolytic activity against cancer cells. The present study investigated the effects of oncolytic NDV strain AF2240 and V4-UPM on osteosarcoma cells (Saos-2) under normoxic and hypoxic conditions.
**Methodology and results:** Results showed that the NDV strain AF2240 and V4-UPM could infect and kill normoxic and hypoxic Saos-2 cells equally well by inducing hypoxia-independent apoptosis, and S-phase cell cycle arrest under the microscopy examination, cell viability assay, Annexin V apoptosis assay and cell cycle analysis experiments. However, the Velogenic NDV strain AF2240 excelled over the lentogenic NDV V4-UPM with increased oncolytic effects in Saos-2 cells.
**Conclusion, significance and impact of study:** In a nutshell, normoxia or hypoxia microenvironment has little effect on NDV-induced oncolysis of Saos-2 cancer cells which poses as a potential agent for the treatment of resistant cancer.

*Keywords:* Hypoxia, Newcastle disease virus (NDV), oncolytic activity, Saos-2 cell

## INTRODUCTION

Cancer is the second leading cause of non-communicable disease-related death worldwide (Bray *et al.*, 2018) after cardiovascular disease (heart attack and stroke). The trend for new cancer data records is expected to increase every year and rise to 28.4 million by 2040 (Sung *et al.*, 2021). This includes osteosarcoma, a highly metastatic bone cancer type usually affecting children and adolescents (Wittig *et al.*, 2002; Damron *et al.*, 2007; Whelan and Davis, 2018). Although osteosarcoma is highly malignant, its number of documented records was found to be much lower than that of other cancer types in the past three decades. This results in limited information on osteosarcoma development and mutation, which eventually increases the mortality rate of osteosarcoma patients due to poor prognosis (Isakoff *et al.*, 2015; Bishop *et al.*, 2016).

Osteosarcoma patients mostly suffer from the recurrence of the disease after surgical resection. This has led physicians to routinely advise patients to undergo chemotherapy sessions before and after the surgery procedure. Unfortunately, the hypoxic environment of osteosarcoma not only promotes the growth of cancer cells but also reduces the effectiveness of chemotherapy, resulting in chemoresistance acquisition (Adamski *et al.*, 2013; Roncuzzi *et al.*, 2014; Zhao *et al.*, 2016; Ma *et al.*, 2017). Hypoxic environments are typical in many types of cancers, which aggressively consume oxygen during growth and create a lower oxygen environment called hypoxia. Thus, alternative treatments are warranted to overcome such obstacles.

Oncolytic virotherapy has been proposed as a new cancer therapy due to its superior anticancer activity against numerous cancer cell lines (Wu *et al.*, 2014; Sharma *et al.*, 2017; Bommareddy and Kaufman, 2018).

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Among the previously reported oncolytic viruses, Newcastle disease virus (NDV) has gained increasing attention due to its cancer-selective oncolytic activity (Kalyanasundram *et al.*, 2018). NDV is an avian virus that belongs to the *Orthoavulavirus* genus and Paramyxoviridae family. The pathotypes of the virus range from the most virulent velogenic strain (AF2240) to the least virulent lentogenic strain (V4-UPM). These strains have been employed frequently in the past to demonstrate the oncolytic activity in cancer cells (Aishah *et al.*, 2012; Assayaghi *et al.*, 2016; 2019; Ahmad *et al.*, 2020). Additionally, previous studies reported that NDV could induce oncolysis in both normoxic and hypoxic cancer cells while leaving normal cells unharmed (Ch'ng *et al.*, 2013; Abd-Aziz *et al.*, 2016). These findings suggest that NDV could be a potential candidate as an alternative cancer therapy in overcoming the hypoxic environment of osteosarcoma. On this account, the present study is designed to compare the oncolytic properties possessed by both velogenic (AF2240) and lentogenic (V4-UPM) strains of NDV in osteosarcoma cells, Saos-2, in both normoxic and hypoxic environments.

## MATERIALS AND METHODS

### Cell line, NDV and culture conditions

The osteosarcoma cell line, Saos-2, was maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS; PAA, Pasching, Austria) at 37 °C in a humidified $CO_2$ incubator. The cells were subcultured three times at $1 \times 10^4$ cm$^{-2}$ (Abd-Aziz *et al.*, 2015), followed by seeding at $2 \times 10^4$ cm$^{-2}$ for the infection study. After overnight incubation, cells infected with either a velogenic NDV strain, designated as AF2240 (Yusoff and Tan, 2001) or a lentogenic strain, designated as V4-UPM (Alabsi *et al.*, 2011), as described previously (Chia *et al.*, 2012; Ch'ng *et al.*, 2015). Time zero or 0 h post-infection began after a 1 h virus adsorption period. The infected cells were subsequently placed in a humidified $CO_2$ incubator for normoxia (21% $O_2$) or a Galaxy 48R incubator (New Brunswick) for hypoxia (0.5% $O_2$).

### Microscopy examination

Fluorescence microscopy (NIKON, Japan) was used to analyse Saos-2 cells after infection. Figures representative of cells were obtained at three time points [24, 48 and 72 h post-virus infection (hpi)] to examine the cytopathic effects of post-virus infection.

### Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is frequently used to assess the viability of cells following exposure to various treatments (Kosni *et al.*, 2016; Leong *et al.*, 2020). Briefly, Saos-2 cells ($2 \times 10^4$ cm$^{-2}$) in a 96-well plate were infected with AF2240 and V4-UPM as described above. At designated time point, all wells were added with 100 µL of MTT solution (0.5 mg/mL) and further incubated for 4 h. After removing the supernatant, 100 µL of dimethyl sulfoxide (DMSO) was added to solubilise the purple formazan crystal and the absorbance was measured at a wavelength of 570 nm with a microplate reader (Bio-tek instruments, USA).

### Annexin V apoptosis assay

Virus induced apoptosis in Saos-2 cells was analysed using Annexin V (BD Biosciences; San Jose, CA, USA) with some modification (Keshavarz *et al.*, 2020). The infected Saos-2 cells were trypsinised at various time points and combined with the spent media. The mixture was then centrifuged at 1500× *g* for 5 min. The pellet was washed with PBS containing 1% FBS (PAA, Pasching, Austria) and underwent another centrifugation round. The resulting pellet was then resuspended with a working buffer containing PE Annexin V and 7-AAD. The mixture was incubated for 15 to 20 min at room temperature, followed by flow cytometry analysis (ACEA, Novocyte). About 10,000 events per sample were gated for analysis.

### Cell cycle analysis

Cells cycle analysis of the infected cells was analysed according to Leong *et al*. (2020). At the designated time point, the cells were harvested and mixed with the spent medium. After centrifugation at 1500× *g* for 5 min, the resulting pellet was fixed with 1 mL of cold 70% ethanol overnight. Next, the solution was centrifuged again at 1500× *g* for 5 min and the pellet was washed with 1 mL of PBS, followed by another round of centrifugation. The supernatant was discarded, and the pellet was again resuspended with 1 mL of PBS. RNAse (50 µL; 1 mg/mL) was added and incubated for 15 min, followed by 20 µL of propidium iodide (1 mg/mL). After 20 min of incubation, the mixture was analysed using flow cytometry (ACEA, Novocyte), in which 10,000 events were gated for analysis.

### Statistical analysis

The data was presented as statistical means +/- standard error of the mean (S.E.M) by using the student's t-test (GraphPad Sofware., La Jolla, CA, USA). Statistically significant data was considered when $p<0.05$.

## RESULTS AND DISCUSSION

The increasing number of cancer cases is a significant issue worldwide (Bray *et al.*, 2018; Sung *et al.*, 2021), despite the availability of the current treatment. To date, chemotherapy (DeVita and Chu, 2008; Makin, 2018), radiation therapy (Delaney *et al.*, 2005; Barker *et al.*, 2015) and surgery (Sullivan *et al.*, 2015) remain the standard treatments for the management of cancers. Although such treatments effectively control early stage

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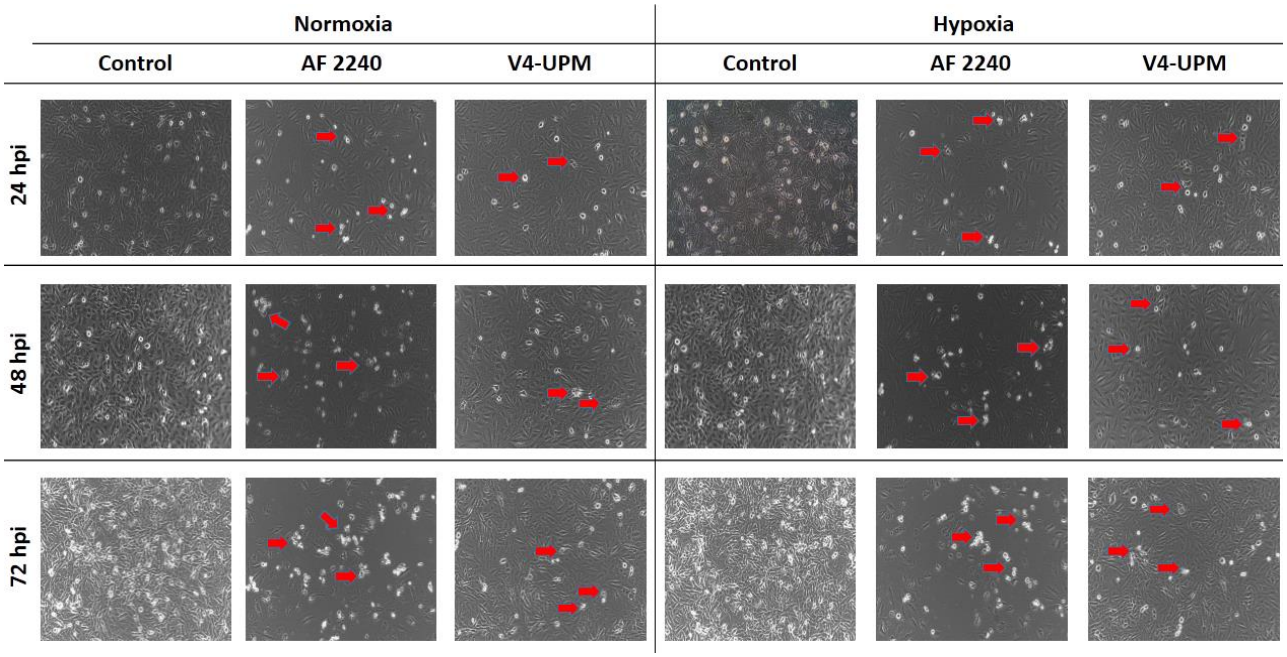


**Figure 1:** Microscopic examinations of NDV-infected Saos-2 cells in normoxia and hypoxia. The figures above were representatives of Saos-2 cells infected by AF2240 and V4-UPM at 24, 48 and 72 hpi. The red arrow indicated the CPE of the NDV-infected cells. All the pictures were taken at 40× magnification.

cancer progression, their associated side effects due to poor tumour selectivity and chemoresistance development remain a major obstacle preventing their full therapeutic potential (Chen, 2000; Chakraborty and Rahman, 2012). Moreover, the recurrence of cancer commonly happens in patients with late-stage cancer, which implied the ineffectiveness of current therapies in treating late-stage cancers (Ciombor and Bekaii-Saab, 2013; Grob *et al.*, 2016). Therefore, novel and effective alternative cancer therapies, including oncolytic virotherapy, are urgently needed to minimize the risk of cancer recurrence and improve the survival rate of cancer patients. NDV is an avian virus that is not pathogenic to humans. It has been reported to be a tumour-selective oncolytic virus. The present study further investigated the anticancer properties of NDV on osteosarcoma cells Saos-2.

**AF2240 and V4-UPM infections induced cytopathic effects on Saos-2 cells**

Qualitative microscopic analysis showed that AF2240 and V4-UPM induced cytopathic effects on Saos-2 cells in a time-dependent manner. The infected cells initially experienced shrinkage of the structure, rounding up and finally, a loss of adherence from the surface of attachment. In both treatment groups, this effect was consistently observed in each time-point 24, 48 and 72 h post-infection (hpi) (Figure 1). However, it was more prominent in the AF2240-infected Saos-2 cells compared to that of the V4-UPM with more floating cells. Interestingly, there was no difference in cell killing for both normoxia and hypoxia conditions. Both NDVs manage to manipulate, especially the hypoxic environment which could possibly cause the cell-killing reaction would be the same as in the normoxia. In return, these observations indirectly suggest that the treatment constraints brought on by the decreased oxygen content (hypoxia) were addressed by AF2240 and V4-UPM. A similar observation was reported by Abd-Aziz *et al.* (2016) on a different cell line in breast cancer in which AF2240 was capable of induced cell lysis in both hypoxic and normoxic conditions.

**AF2240 and V4-UPM infections reduced the viability of Saos-2 cells**

To further quantify the cytopathic effects of viruses on Saos-2 cells, MTT assay was conducted to evaluate the effects of AF2240, and V4-UPM on Saos-2 cell viability, and the results are summarised in Figure 2. Based on the results obtained, infection with V4-UPM has reduced the cell viability of Saos-2 to 65.37% at 24 hpi under a normoxic environment. The viability percentage was further dropped to 54.21% and 53.61% when the treatment was prolonged to 48 and 72 hpi, respectively. Similar observations were also made in AF2240 infected cells in which the cell viability was reduced drastically from 56.73% at 24 hpi to 44.58% and 18.27% at 48 and 72 hpi, respectively. These observations are consistent with our previous microscopic inspection analysis in which we discovered that AF2240 possessed more prominent

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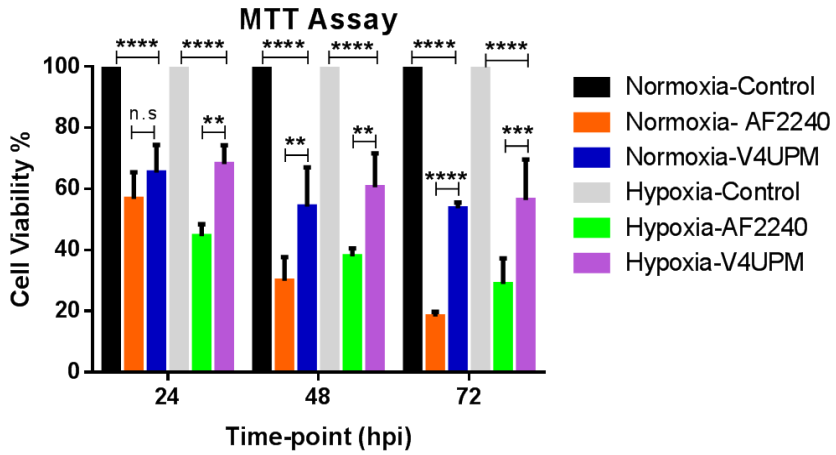


**Figure 2:** Cell viability of NDV-infected Saos-2 cells in normoxia and hypoxia. The bar graph shows that AF2240 and V4-UPM infected Saos-2 cells gradually decreased cell viability with increased incubation time for both normoxia and hypoxia conditions. Data presented here were expressed as the mean percentage of cell viability for each type of group. *Indicates the significant values $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, n.s=not significant.

oncolytic activity than V4-UPM. Noteworthy, the oncolytic activity of AF2240 and V4-UPM were found to be independent of oxygen levels as no significant difference in cell viability was detected between the infected hypoxic and normoxic cancer cells. Infections of AF2240 and V4-UPM have drastically reduced the cell viability percentage at all time points, including 24 hpi. This finding was contradictory to our microscopic examination in which we found that the oncolysis in Saos-2 happened only after 48 hpi. Since cytopathic effects were minima, the viability reduction observed at 24 hpi could be due to the growth inhibition in response to virus infection rather than the cell death induced by virus infections. Additionally, this trend of results, a gradual decrease in cell viability percentage over the incubation period, could be observed from a previous study using AF2240 towards several types of human breast cancer cells, namely MDA-MB231 and MCF-7 (Mohamed Amin *et al.*, 2019). Meanwhile, AF2240 was found to be a better oncolytic virus due to its stronger cytopathic effects and greater cell viability reduction activity on Saos-2 cells. This result complies with a previous study (Alabsi *et al.*, 2011), which showed that the AF2240 requires a lower concentration of virus compared to the V4-UPM to kill half the leukaemia cells in the respective experiment.

**AF2240 and V4-UPM infections induced hypoxia-independent apoptosis Saos-2 cells**

In order to investigate the possible mechanism induced by AF2240 and V4-UPM in Saos- 2 cell, Annexin V-based flow cytometry analysis was performed. Annexin V is a $Ca^{2+}$-dependent phospholipid-binding protein that has a high affinity for the anionic phospholipid phosphatidylserine (PS). When apoptosis is initiated, PS located in the cytoplasmic surface will be translocated to the surface of the plasma membrane outer layer. This translocation allowed Annexin V to bind with PS, thus making Annexin V a helpful agent in detecting apoptotic events in cells (Hingorani *et al.*, 2011; Zembruski *et al.*, 2012). Annexin V-based flow cytometry analysis was performed to evaluate the apoptosis inducing ability of AF2240 and V4-UPM at 24, 48 and 72 hpi, and the result obtained are summarised in Figure 3. At 24 hpi with AF2240 and V4-UPM, the cell populations of the density plots' right and upper right quadrants were increased. Although the increment was relatively low, it is sufficient to suggest that AF2240 and V4-UPM were capable of inducing the onset of apoptosis in both normoxic and hypoxic Saos-2 cells as early as 24 hpi. When the treatment was prolonged to 48 and 72 hpi, the total apoptotic cells detected in AF2240 infected Saos-2 was drastically increased from 6.98-8.60% (24 hpi) to 40.10-42.63% and 55.17-55.31%, respectively, indicating the progression of apoptosis. A similar trend but with a lower magnitude was observed in V4-UPM-infected Saos-2 cells in which its apoptotic percentage rose from 2.62-4.43% at 24 hpi to 14.94-15.2% and 28.22-37.8% at 48 and 72 hpi, respectively.

Only a small number of apoptotic cells are detectable at 24 hpi while high levels of cell apoptosis have been detected at both 48 and 72 hpi. This finding, in turn, confirmed that the cell viability reduction at 24 hpi observed in the MTT assay was not caused by cell death but rather resulted from growth inhibition induced by the virus infections. The relatively low activities (absence of cytopathic effects and low levels of cell apoptosis) observed at 24 hpi could be due to the short incubation period, which was insufficient for 1 MOI of viruses to complete their lytic cycle. Noteworthy, the effects of hypoxia on apoptosis inducing ability of both viruses appeared to be minimal, as similar apoptotic levels were detected in both infected normoxic and hypoxic cancer cells. The current data confirmed that AF2240 and V4-UPM could induce hypoxia-independent apoptosis in Saos-2 cells.

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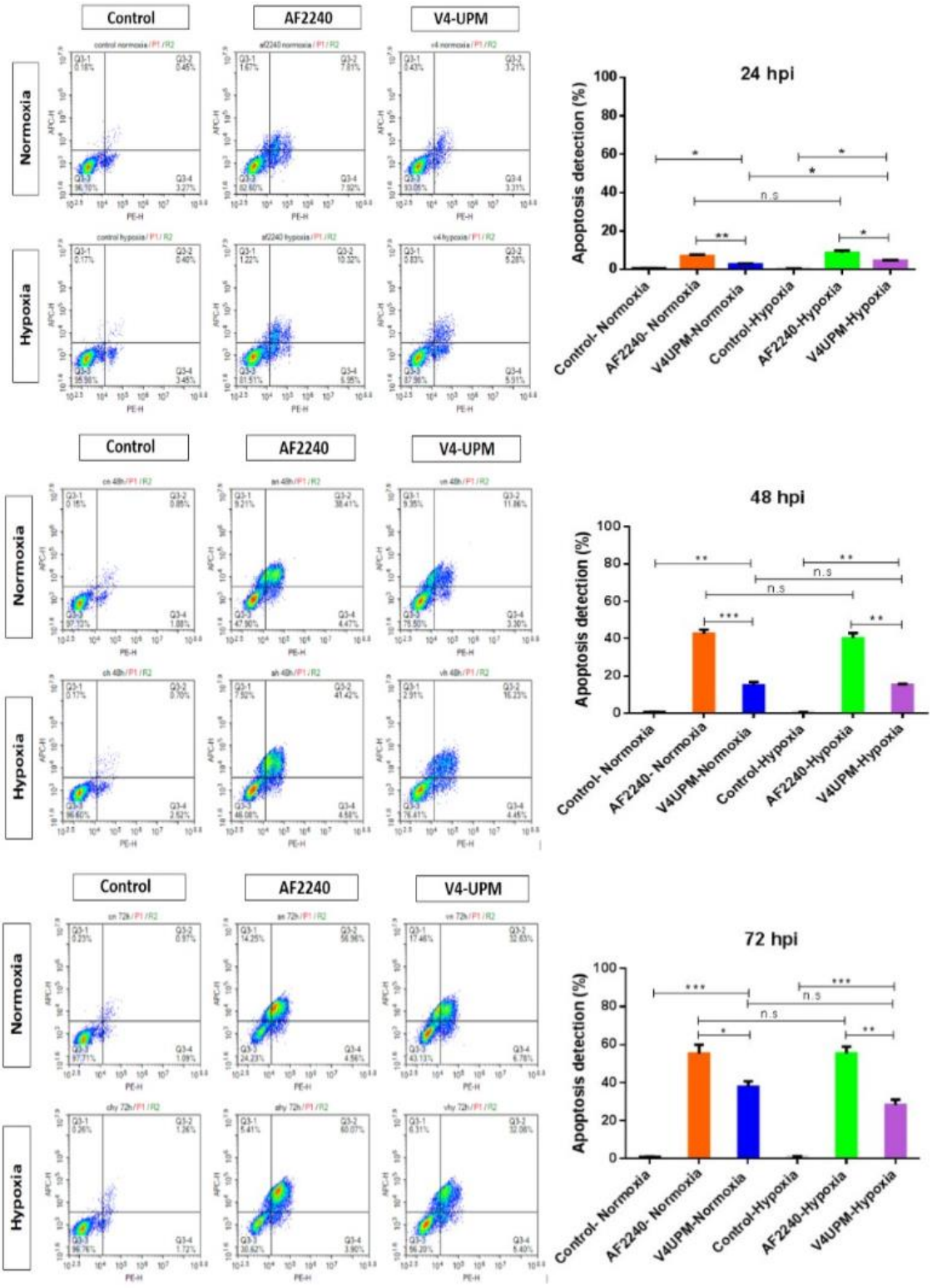


**Figure 3:** PE Annexin V apoptosis assay. This assay showed that NDV AF2240 treatment produced more apoptosis than V4-UPM towards the Saos-2 cell at each time point under both conditions. Data presented here represented experiments performed in triplicate. n.s=not significant, **p*<0.05, ***p*<0.01, ****p*<0.001.

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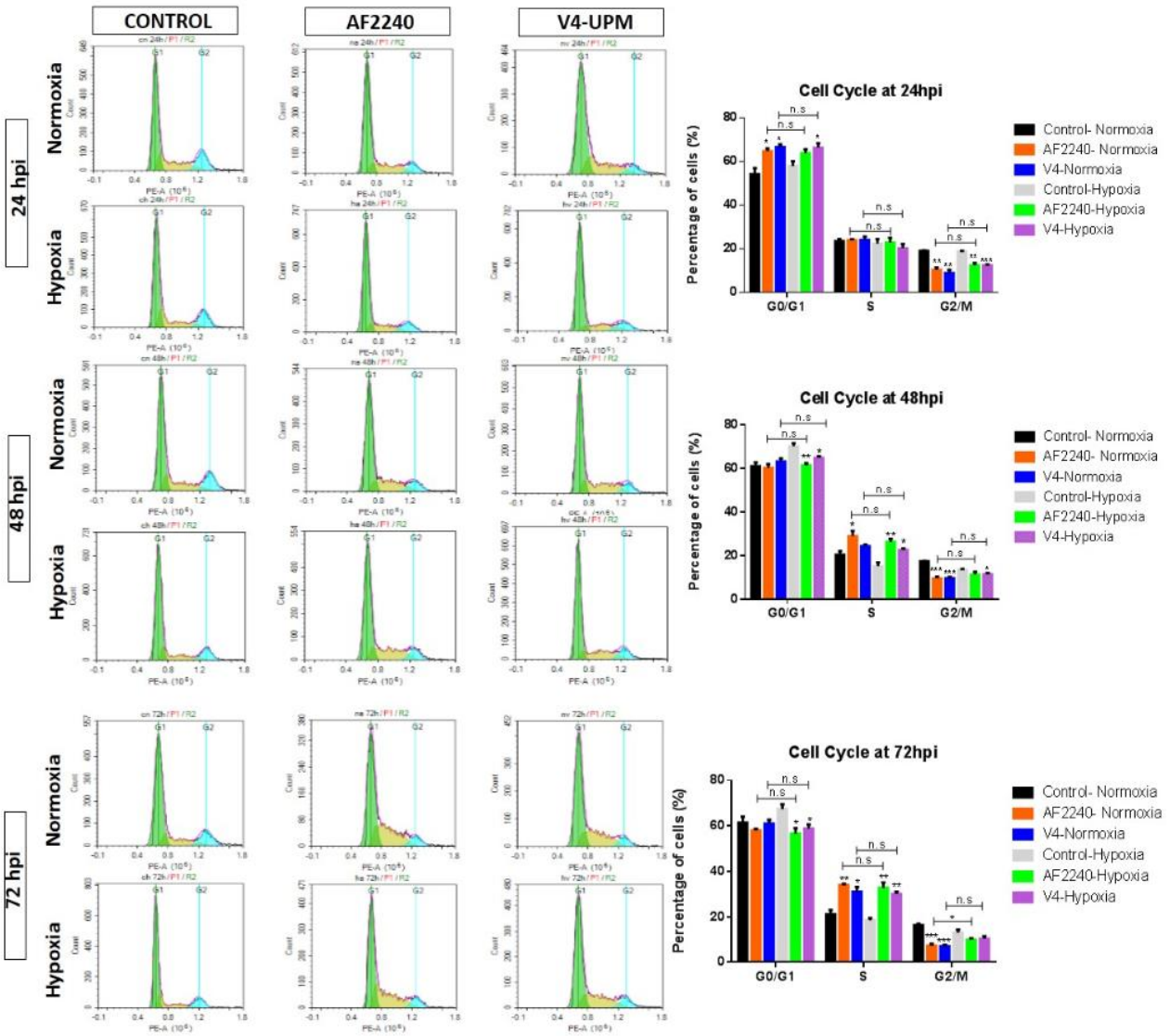


**Figure 4:** Cell cycle analysis of NDV-infected Saos-2 cells at various time points in normoxia and hypoxia. The cells were stained with propidium iodide (PI) and analysed by NovoCyte ACEA Bioscience flow cytometry. Data presented here were the mean value of the percentage of cells in which all the significant values were present after being compared with the respective control. n.s=not significant, **p*<0.05, ***p*<0.01, ****p*<0.001**.**

**AF2240 and V4-UPM induced G0/G1-phase arrest at 24 hpi and S-phase arrest at 48 and 72 hpi in Saos-2 Cells**

Cell cycle analysis was conducted to study the effects of AF2240 and V4-UPM on cell cycle progression in Saos-2 cells at different time points, 24, 48 and 72 hpi (Figure 4). The results showed that both AF2240 and V4-UPM induced cell cycle arrest in the infected Saos-2 cells. The cell populations were increased and decreased at G0/G1 and G2/M, respectively. This suggested that both AF2240 and V4-UPM could induce G0/G1 cell cycle arrest in Saos-2 cells at 24 hpi. This finding is in consistence with previously reported data in which Wang and co-workers (2018) reported that NDV infection induces G0/G1-phase arrest to facilitate its viral replications at the early stage of infection (Wang *et al.*, 2018). Surprisingly, when the treatment was prolonged to 48 and 72 hpi, both viruses were found to induce S-phase arrest in the infected cells as the cell population at the S-phase was increased reciprocally to the decrease of the G2/M peak. This could be due to the maturity of the cellular viral particles, which eventually will cause DNA damage in the infected cells, resulting in S-phase arrest and apoptosis. This explanation is comparable to the earlier work on the cell cycle, where treating the mammalian cells with hepatitis C

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virus caused cell growth inhibition through downregulation of the expression of cyclin A protein, involved in the S-phase of the cell cycle (Yang *et al.*, 2006). Besides, similar reports on the S-phase cell cycle arrest due to viral infection could be obtained from previous studies (Trapp-Fragnet *et al.*, 2014; Yu *et al.*, 2015; Liu *et al.*, 2022). Others, both viruses exhibited similar effects independent of the hypoxic treatment condition.

NDV strain AF2240 is a better oncolytic virus due to its stronger oncolytic activity in all experiments performed in this study. AF2240 is a velogenic strain of NDV containing F protein with polybasic cleavage site, which was cleaved into its active form during post-translational modification and is ready for new infection cycle. On the other hand, the lentogenic V4-UPM contains F protein with monobasic cleavage sites (Heiden *et al.*, 2014) which require additional enzymatic digestion to activate the F protein for a fusion of the virus into the cell. Therefore, it was presumed that the Velogenic NDV has better oncolytic activity compared to the lentogenic NDV. The present data showed that AF2240 and V4-UPM could kill normoxic and hypoxic Saos-2 cells by inducing hypoxia-independent apoptosis and S-phase cell cycle arrest.

The effect of different microenvironment settings seems to be not affected in this study which may be due to oncolytic activity can vary depending on the specific oncolytic virus and cancer cell type being studied. Previous studies have reported that hypoxia reduces the efficiency of replication and oncolysis during group B and C adenovirus infections toward several types of cancer cells (Pipiya *et al.*, 2005; Shen and Hermiston, 2005; Shen *et al.*, 2006). Conversely, establishing pancreatic cancer cells with hypoxia prior to H-1 parvovirus infection resulted in increased viral replication followed by cell death induction (Cho *et al.*, 2015). From here, further research is needed to better understand the mechanisms underlying the interaction between oncolytic virus and hypoxia in cancer cells.

## CONCLUSION

The current study illustrated a better oncolytic activity of NDV strain AF2240 compared to that of the lentogenic V4-UPM strain in both hypoxic and normoxic conditions by inducing hypoxia-independent apoptosis and S-phase cell cycle arrest. Despite all the significance and positive results obtained from these experiments, a more in-depth study should be carried out to further investigate the effect of pathotypes on the oncolytic activity of the virus. The insignificant differences in NDV-induced oncolysis between hypoxic and normoxic conditions suggested that NDV could overcome the obstacle faced by many chemotherapeutic agents in hypoxic conditions in killing the cancer cells. It would be interesting to investigate the underlying process of cell death in a hypoxic environment induced by different NDV pathotypes, which warrant further study.

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