Oncolytic Newcastle disease virus effects on immune response – a new issue in cancer treatment

Účinky onkolytického viru Newcastleské choroby na imunitní odpověď – nová problematika v léčbě nádorových onemocnění

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Summary

Background: Millions of people are diagnosed with cancer each year, and fighting it puts a heavy financial burden on communities and governments. Numerous advances have been made in the field of cancer; one of the newest methods is using oncolytic viruses. This study aimed to evaluate the effect of oncolytic Newcastle disease virus wild-type strains (NDV-WTS) on the immune system. ***Material and methods:*** Forty mice were divided into four groups (10 animals in each group). The control group received phosphate buffered saline, and experimental group 1 (NDV-WTS 1), experimental group 2 (NDV-WTS 2), and experimental group 3 (NDV-WTS 3) received 10^{-1}, 10^{-2}, and 10^{-3} titers of Newcastle virus on 0, 14th, and 28th days. On the 31st day, 100 µL of Newcastle virus was injected into the left footpads of animals. After 48 hours, delayed-type hypersensitivity (DTH) reactions were measured. On the 33rd day, peritoneal macrophages were isolated. Then proliferation of the cells was measured by the methyl-thiazolyl-tetrazolium (MTT) test. Neutral red uptake and respiratory burst of peritoneal macrophages were also assessed. Data were analyzed using statistical software SPSS, version 19. ***Results:*** The results of the DTH test showed that footpad swelling in control, NDV-WTS 1, NDV-WTS 2, and NDV-WTS 3 groups were 23.5%, 23.5%, 23.6% and 23.6%. No significant differences were seen between the groups in this regard ($P > 0.05$). A negative nitroblue tetrazolium (NBT) reduction test as an indicator of macrophage's respiratory burst, showed no significant difference between the groups ($P > 0.05$). The neutral red uptake assay and MTT test showed no significant differences between the groups ($P > 0.05$). ***Conclusion:*** The results of this study showed that NDV-WTS in doses of 10^{-1}, 10^{-2}, and 10^{-3} have no adverse effects on healthy normal cells.

Key words

cancer – virus – Newcastle virus – immune system – oncolytic virus – mice

The authors declare that they have no potential conflicts of interest concerning drugs, products, or services used in the study.

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Souhrn

Východiska: Každý rok jsou milionům lidí diagnostikována nádorová onemocnění a jejich léčba představuje pro plátce zdravotní péče finanční zátěž. V oblasti nádorových onemocnění byl učiněn velký pokrok; jednou z nejnovějších metod je využití onkolytických virů. Cílem této studie bylo hodnocení vliv divokých kmenů onkolytického viru Newcastleské choroby (Newcastle disease virus wild-type strains – NDV-WTS) na imunitní systém. ***Materiál a metody:*** Čtyřicet myší bylo rozděleno do čtyř skupin (10 zvířat v každé skupině). Kontrolní skupině byl aplikován fosfátový pufr a experimentálním skupinám 1 (NDV-WTS 1), 2 (NDV-WTS 2) a 3 (NDV-WTS 3) byl 0., 14. a 28. den aplikován Newcastleský virus v titrech 10^{-1}, 10^{-2} a 10^{-3}. Třicátý první den bylo zvířatům do levého chodidla vpíchnuto 100 µl Newcastleského viru. Po 48 hodinách byla měřena přecitlivělost oddáleného typu (delayed-type hypersensitivity – DTH). Třicátý třetí den byly izolovány peritoneální makrofágy. Pak byla měřena proliferace buněk pomocí methyl-thiazolyl-tetrazolium (MTT) testu. Rovněž bylo hodnoceno vychytávání neutrální červeně a respirační vzplanutí makrofágů. Data byla analyzována pomocí statistického software SPSS, verze 19. ***Výsledky:*** Výsledky DTH testu ukázaly otok chodidla u kontrolní skupiny a ve skupinách NDV-WTS 1, NDV-WTS 2 a NDV-WTS 3 z 23,5 %, 23,5 %, 23,6 % a 23,6 %. V tomto ohledu nebyly mezi jednotlivými skupinami významné rozdíly ($p > 0,05$). Negativní nitroblue tetrazolium (NBT) test jakožto indikátor respiračního vzplanutí makrofágů neprokázal mezi skupinami významné rozdíly ($p > 0,05$). Vychytávání neutrální červeně a MTT test rovněž neukázaly mezi skupinami významné rozdíly ($p > 0,05$). ***Závěr:*** Výsledky této studie svědčí o tom, že DV-WTS v dávkách 10^{-1}, 10^{-2} a 10^{-3} nemají na normální zdravé buňky žádné vedlejší účinky.

Klíčová slova

nádorová onemocnění – virus – Newcastleský virus – imunitní systém – onkolytický virus – myši

Introduction

Millions of people are diagnosed with cancer yearly, and fighting it puts a heavy financial burden on communities and governments. Numerous advances have been made in the field of cancer, including improvements in early detection techniques, surgery, and chemotherapy, hormone therapy, immunotherapy, gene therapy, and radiation therapy [1]. Despite all these advances, cancer remains the second leading cause of death in the United States [2].

Over the past few decades, advances in cancer genetics have led to the development of new targeted therapies for cancer, such as monoclonal antibodies. Although targeted molecular therapies have gained credibility, newer therapeutic approaches are needed. Many human viruses have been evaluated for selectively infecting, proliferating, and killing cancer cells. Viruses plays an important role in the treatment process [3,4]. Due to the nature of the virus, how it is transmitted and infected, and how it proliferates, the therapeutic approach to using oncolytic viruses (OVs) in cancer was developed. While infecting and killing tumor cells (oncolysis), OVs are viral strains that do not damage normal cells.

The new era of virus therapy began in the early 1990s with the injection of attenuated herpes simplex virus (HSV) into the mouse glioma model [5]. The virotherapy then progressed from the laboratory to clinical trials. To date, many viruses have been identified in humans and animals that have been examined for their anti-cancer potential and immunity. These viruses include adenoviruses, HSV, measles, rheumatoid arthritis, lentivirus, parvovirus, mumps virus, and smallpox virus [5,6].

Some of the main oncolytic viruses mechanisms include the proliferation of viruses in cancer cells, the presence of some toxic proteins in the virus that destroy the cancer cell, stimulate and activate the immune systems of the virus against cancer cells, and ultimately angiogenesis in cancer cells that destroy them [7]. Newcastle disease virus (NDV) belongs to the genus *Avulavirus*, family *Paramyxoviridae*, and causes severe disease in birds. Because it is non-pathogenic in mammals, it is a good candidate for cancer treatment [8].

New studies have shown the ability and persistence of NDV in the human body. The problem with many oncolytic viruses is the negative reaction of the human body to them. However, in 96% of cases, the human body has been seronegative to this virus. Despite all the advantages mentioned, clinical trials have not been satisfactory [9,10]. The virus responds to cancers in various ways. Perhaps by interfering with the immune system, its ability is reduced. Therefore, the present study was performed to evaluate the effect of oncolytic Newcastle disease virus wild-type strains (NDV-WTS) on immune response in Balb/c mice.

Material and methods

Study design

This experimental study was conducted at Applied Virology Research Center, Baqiyatallah University of Medical Science, Tehran, Iran. Forty Balb/c mice (weight 30–50 grams) were prepared from the Animal Center at Baqiyatallah University of Medical Science. The animals were kept in plastic cages with 12/12 h light/dark cycle at a normal condition.

Studied groups

Forty mice were divided into four groups (10 animals in each group). The control group received phosphate buffered saline (PBS) IP on the 0, 14th, and 28th days. Experimental group 1 received a titer of 10^{-1} Newcastle virus IP on the 0, 14th, and 28th days. Experimental group 2 received a titer of 10^{-2} Newcastle virus IP on the 0, 14th, and 28th days. Experimental group 3 received a titer of 10^{-3} o Newcastle virus IP on the 0, 14th, and 28th days.

Experimental tests

Newcastle virus was received at titers 10^{-1}, 10^{-2}, and 10^{-3} from Applied Virology Research Center, Baqiyatallah University of Medical Science, Tehran, Iran. Injecting the viruses was done on the 0, 14th, and 28th days. The mice's weight was measured on the 0, 14th, and 33rd days.

Cellular immunity

On the 31st day, 100 µL of Newcastle virus (titer for each group similar to intraperitoneal injection) was injected into the left footpads of animals. At the same time, PBS was injected into the right footpads of animals. After 48 hours, delayed-type hypersensitivity (DTH) reac-

tions represented by the degree of footpad swelling were measured using a Mauser dial caliper (Germany). The level of cellular immunity was calculated according to the following formula:

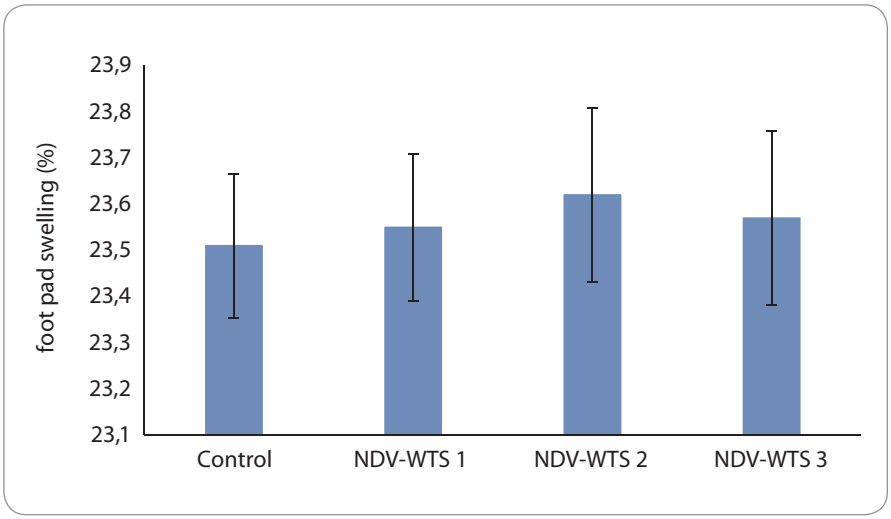
$$\text{Cellular immunity index} = \frac{\text{swelling of the left foot} - \text{swelling of the right foot}}{\text{swelling of the right foot}}$$

Isolation of peritoneal macrophages

At the end of the study period, all mice were anesthetized. Then 8 mL of cold sterile phosphate buffer was injected into the abdominal cavity of mice. After injection and slow massage to release the cells, the injected fluid was sucked out of the mice peritoneum with a syringe. The resulting liquid was transferred to a test tube. The cells were washed three times with 4 °C phosphate buffer. After cell count, the cell suspension was added to 24-well in a number of one million cells in DMEM culture medium and incubated for 2 hours at 37° C and 5% CO_2. The external supernatant and wells were washed three times with phosphate buffer to remove non-adhesive cells. The cells that remained attached to the bottom of the flask were mostly macrophages.

Preparation of spleen cell culture and cell proliferation test

The spleens of mice were removed and weighed under sterile conditions. The spleen tissue was then shredded and crushed in 5 mL of RPMI-1640 culture medium (Sigma Company, USA) containing 10% fetal bovine serum (FBS) (Gibco Company, Germany). The tissue was passed through a mesh with a diameter of 0.2 mm to prepare a cell suspension. After centrifugation for 10 minutes at 2,000 rpm, 5 mL of lysis buffer was added to the resulting cell precipitate to remove red blood cells. After 5 minutes, adding 10 mL of culture medium was centrifuged again at 2,000 rpm for 10 minutes. Cell precipitation was then suspended in RPMI culture medium containing 10% FBS. Following cell count, a suspension containing 1×10^6 cell/mL was prepared, and 100 µL of it was poured into each 96-well plate. For each sample, three replications without Newcastle and three replications in the presence of 50 µL of phytohemagglutinin solution (1 mg/mL) were considered. An empty RPMI medium was used as a blank in three wells. After 72 hours of incubation under 5% CO_2 condition, 25 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for other 4 hours. During this period, reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) by living and proliferating cells resulted in the formation of formazone crystals, which was dissolved by adding 100 µL of DMSO. Then the color intensity was determined at 490 nm.



Graph 1. Comparison of cellular immunity index in the studied groups.
NDV-WTS – Newcastle disease virus wild-type strain

Neutral red uptake assay for peritoneal macrophage

Inside the well containing macrophage in a ratio equal to the culture medium, 33% neutral solution (0.03333 g of neutral powder dissolved in one mL of culture medium) was incubated for 2 hours. The supernatant was then collected and the volume of the initial amount of 1% acetic acid (one gram of acetic acid in 50 mL of ethanol and 50 mL of distilled water) was added. 200 µL per well was poured from the 96-well microplate. Finally, the results of the work were read by ELISA reader at 492 nm.

Respiratory burst of peritoneal macrophages

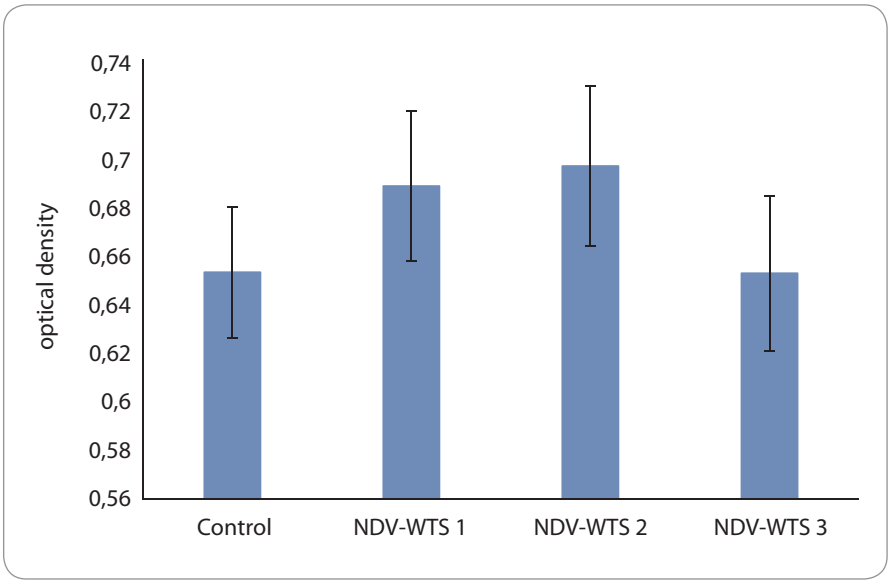
Negative nitroblue tetrazolium (NBT) reduction test was used in this step. Half a mL of zymosan plus half a mL of 0.1% NBT solution (2 mg of 0.1% NBT yellow powder dissolved in 2 mL of PBS buffer) were added to the well containing macrophages and incubated at 37 ° C for 30 minutes. After incubation, the cells were separated and centrifuged at 2,000 rpm for 10 minutes. Remove a small amount of the top layer with a sampler, add three drops of 70% methanol, wait 10 minutes for the alcohol to dissolve, and then make up to 1.5 mL with PBS buffer and pipette well. In each cell of the 96-cell plate, 200 µL of the solution was added. Now 100 µL of 2M KOH and 200 µL of DMSO have been added to each cell and shaken with pepting. Finally, the optical density was read by ELISA reader at 492 nm.

Statistical analysis

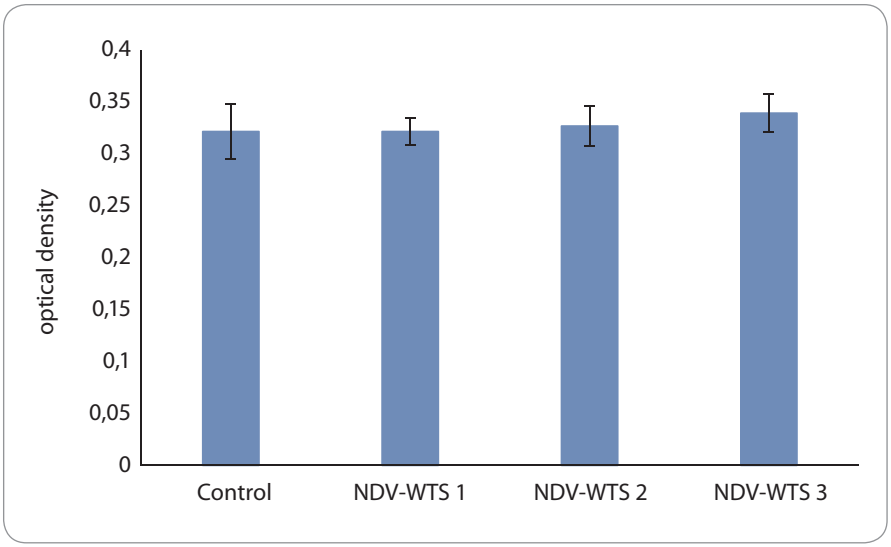
All statistical analyzes were performed in SPSS software version 19 using one-way ANOVA and LSD backup tests. Microsoft Excel (2010) software was used to draw the graphs. All data were reported as mean ± SD. $P < 0.05$ was assumed as statistically significant.

Results

Forty mice were divided into four groups (10 animals in each group). The control group, experimental group 1 (NDV-WTS 1), experimental group 2 (NDV-WTS 2), and experimental group 3 (NDV-WTS 3).



Graph 2. Respiratory burst of peritoneal macrophages in the studies groups.
NDV-WTS – Newcastle disease virus wild-type strain



Graph 3. Neutral red uptake assay for peritoneal macrophage in the studies groups.
NDV-WTS – Newcastle disease virus wild-type strain

To find the possible effects of NDV-WTS on cellular immunity, the effects of this virus on DTH was evaluated. The footpad swelling in control, NDV-WTS 1, NDV-WTS 2 and NDV-WTS 3 groups were 23.5%, 23.5%, 23.6%, and 23.6%. According to our analysis, no significant difference was seen between the groups in terms of DTH ($P > 0.05$) (Graph 1).

The negative NBT test measures the ability and capacity of macrophages to produce free radicals. The produced superoxide anion reduces the NBT to an insoluble blue formazan. Blue formazan is known as an indicator of the respiratory burst of macrophages. In this study, the optical density showed no significant difference in the control, NDV-WTS 1, NDV-WTS 2, and NDV-WTS 3 groups ($P > 0.05$) (Graph 2).

Neutral red uptake assay for peritoneal macrophage was used in this study. The statistical analysis showed no significant differences between the control, NDV-WTS 1, NDV-WTS 2 and NDV-WTS 3 groups ($P > 0.05$) (Graph 3). The increase or decrease in living cells due to mitochondrial activity leads to the change in the reduction of MTT to formazone so that cell proliferation can be measured. The statistical analysis showed no significant differences between the control, NDV-WTS 1, NDV-WTS 2, and NDV-WTS 3 groups ($P > 0.05$) (Graph 4).

Discussion

In the present study, we investigate the effects of NDV-WTS on immune response in Balb/C mice. Our results demonstrated that NDV-WTS intraperitoneal injection with 10^{-1}, 10^{-2}, 10^{-3} doses and 0, 14, and 28 time-points have no significant effect on immune system responses between the NDV-WTS -treated groups and the control group.

Based on the literature review, we realized that they are not only NDVs pathogenic for mammals but they also have natural oncolytic effects, which highlight them as anticancer agents. Wild-type NDV strains are safer and more cost-effective than modified recombinant strains that require extra measures for genetic control of viral products [11].

It has been proven that the NDV plays an essential role in immune system stimulation, and its various strains can stimulate immune system responses in cancer cells of different species [12–15]. For example, Yurchenko et al (2018) demonstrated that all cancer cell lines, but not healthy and normal cells, had different degrees of immunostimulatory to wild-type NDV infection [11].

Also, some previous studies showed that NDV exerts oncolytic properties through stimulating cellular innate immune responses in the cells of colorectal, gastric, pancreatic, bladder, breast, ovarian, renal, lung, larynx, and cervical carcinomas, glioblastoma, melanoma, pheochromocytoma, lymphomas of different origins, fibrosarcoma, osteosarcoma, and neuroblastoma, but not normal cells [16–18].

Dmitriy et al (2012) showed that the replication and spread of NDV are significantly reduced in normal cells compared with cancer cells due to antiviral signaling pathways [19], the different expression of sialoglycoproteins on the surface of cells, and interferons [20–22].

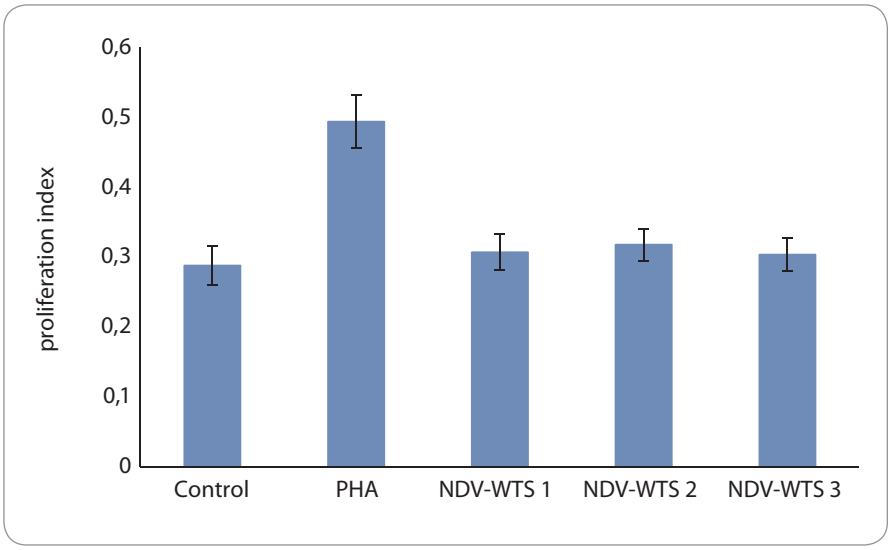
Numerous reports have shown that the virus cannot replicate in non-trans-

formed cells, such as fibroblast cells, resting T lymphocytes, and normal primary culture [23–26]. Our study was in line with the above, emphasizing that NDV does not stimulate the immune system in normal and healthy cells.

Cell infection via NDV involves two steps: 1) cell binding, membrane fusion [27], transduction of the viral genome and transcription of viral genes; 2) viral replication using a newly produced nucleocapsid as anti-genome [28,29]. Normal cell infection generally does not proceed to the second step [28,30]. In contrast, in human/murine tumor cells, NDV infection proceeded to the second step, thus allowing tumor selective replication [23]. Therefore, NDV can replicate up to 104-fold faster in human cancer cells than in most normal human cells [24]. This issue could explain the ability of NDV to stimulate the immune system in cancer cells as opposed to healthy cells.

There are mechanisms that NDV has been known as an oncolytic agent including: 1) the hemagglutinin-neuraminidase (HN) and fusion glycoproteins of NDV cause the fusion of infected cells with their neighboring uninfected cells, syncytium formation and tumor cell death [31,32]; 2) direct interaction between the HN protein with sialic acid in the cell surface activate natural killer cells that cause cytokines secretion, such as interleukin 2, interferon (IFN) γ, and tumor necrosis factor α, as well as influence and activate function of other immune cells [33]; 3) NDV can activate macrophages and increases their enzymes, such as adenosine deaminase, inducible nitric oxide synthase, lysozyme, and acid phosphatase [34]; 4) IFN-β and IFN-α exhibit antiproliferative effects by p53 induction, CD8+ T lymphocyte and activated macrophage, chemokine secretion, and miR-21 decrease [35]; 5) NDV cause immunogenic cell death that induces endoplasmic reticulum stress, immunogenic apoptosis, necrosis and autophagy [36].

Zamarin et al (2009) revealed that the generation of the NDV with modified recombinant strain resulted in increased viral replication and enhanced oncolysis in various tumor cell lines compared to the wild-type NDV. Also, they found that the immune system's response to NDV varied depending on the type and strain. They speculate that higher doses and administration of longer treatment regimens would result in an even more significant oncolytic effect and survival [16]. In human, it is reported that NDV causes immune system stimulation and mild side effects, which are temporary and clear within 1–2 days after injection. These side effects include conjunctivitis, laryngitis, hypotension, fever (up to 38 °C), chills, tiredness, headache, muscle pain, weakness, leucopenia, neutropenia, and erythema, swelling, induration, and itching on the vaccination sites [37–40].



Graph 4. Comparison of cell proliferation in the studied groups.
PHA – phytohaemagglutinin

According to previous studies, the NDV oncolytic effects were dose- and time-dependent as well as NDV strain and species studied [18]. Perhaps our study could stimulate immune system responses by injecting NDV-WTS at higher doses or closer injection intervals.

Conclusions

In brief, our results indicated that NDV-WTS intraperitoneal injection with 10^{-1}, 10^{-2}, 10^{-3} doses and 0, 14, 28 time-points have no adverse effects on healthy normal cells and cannot stimulate immune system responses. Future studies are needed to evaluate the effects of NDV-WTS on the immune system responses in higher doses or closer injection intervals.

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Author contributions

Conceptualization – WK and HE; methodology – MF, RD; validation – BJ; data curation – MB; writing (original draft preparation) – HE, WK; writing (review and editing) – all. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflicts of interest. The funders had no role in the writing of the manuscript, or in the decision to publish.

Ethics

The study protocol was reviewed and approved by the ethics committee of the Baqiyatallah University of Medical Sciences (IR.BMSU.REC.1398.287). All experimental tests were carried out following the Baqiyatallah University of Medical Sciences Ethical Committee.

Consent to participate

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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