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# The investigation of the efficacy of the prodrug DDI-10 against Newcastle disease virus infection in young chicken

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## Abstract

**Introduction**. Newcastle disease virus (NDV) is the deadliest virus in the poultry industry. Many RNA viruses induce oxidative stress on the host during its pathogenesis, NDV being one among them. The present study aims to evaluate the protective property of novel phosphorylated DDI-10 in vivo in experimentally infected chicken. **Material and methods**. NDV induced oxidative damage in the liver and lung were measured by determining antioxidant enzyme levels, protein oxidation and nitration using ELISA, Western blot and immuno co-localization assay. **Results**. Glutathione dependent enzymes GPx, GST, and GR were significantly decreased in the NDV infected group due to pathogenesis; DDI-10 treatment was shown to significantly increase this reduced enzyme activity. In comparison to the healthy control group, protein oxidation and nitration levels were significantly increased in the NDV infected group. In the DDI-10 treated group, the oxidation and nitration levels were decreased compared to the NDV infected group. Further estimation of protein nitration and oxidation in western blot and immune co-localization assays correlated with the ELISA results; an intensified band was demonstrated in the NDV infected tissue group, in addition to a high number of co-localized cells being present in immunofluorescence-stained sections compared to control chicken tissues. These alterations were noticeably reduced in novel phosphorylated DDI-10 treatment group. **Conclusions**. These results suggest that DDI-10 mitigates NDV induced oxidative stress, subsequently exerting an ameliorative effect against NDV pathogenesis.

**Key word:** newcastle disease virus, oxidative stress, phosphorylated compound, glutathione.

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## Introduction

Newcastle Disease Virus (NDV) is a paramyxovirus that affects an extensive range of avian species, particularly chicken(1). Apart from birds, NDV is also able to infect humans, but it is not as virulent and has been shown to cause flu-like symptoms, conjunctivitis or laryngitis(2). As an RNA virus, NDV replication takes place in the host cell i.e., infected cells. New progenies and proteins of the virus, along with its genetic material, are formed in the host cell's cytoplasm. The progeny virus particles are released from the host cell's cytoplasm by means of viral budding that makes NDV as an enveloped virus(3). Entry of the virus into the host cells is mediated by two unique pathways: direct fusion between the host's plasma membrane and the viral envelope or receptor-mediated endocytosis. Fusion protein(F) and haemagglutinin neuraminidase(HN) are two glycoproteins involved in the NDV infection. The HN protein of NDV has multiple crucial functions: binds the virus to the host cell's membrane using sialic acid receptors, induces the fusion and entrance of the virus into the host cell, assists with virion release, and prevents self-agglutination in order to enhance the spread of the virus(4).

India stands second and third places among egg and poultry meat producers in the world with a market value worth 90,000 crore rupees. Around the world the poultry industry faces the possibility of huge economic losses due to numerous problems caused by disease. Newcastle disease virus in particular poses a major economic threat to poultry farmers due to the disease's high mortality rate in chicken. In spite of rigorous vaccination administration, Newcastle

disease virus still causes tremendous economic loss for poultry farmers(5). In comparison to all other animal viruses, Newcastle disease virus is the leading cause of economic loss worldwide for all types of poultry farmers(6). NDV currently lacks an effective means of treatment; vaccination is recommended strictly for prevention and is in regular practice. Regardless of widely practiced vaccination administration, NDV outbreaks in commercial poultry for most countries are endemic. This poses a major threat to the poultry industry and requires strict measures for prevention and control of the disease(7). Though various vaccination methods are available, outbreaks of NDV have not been controlled. Hence, there is an imperative need to develop new preventive measures to control NDV.

Oxidative stress is implicated as a pathogenic factor in a number of viral infections, subsequently resulting in reactive species to be common in viral infections. An imbalance in the production of reactive species and the inability of the cells to detoxify is known as oxidative stress(8). Protection against these reactive species is facilitated by both enzymatic and non-enzymatic antioxidant defence systems. Unfortunately, these systems are often insufficient at providing protection against abundant oxidative stress. In an array of microbial infections, oxygen radicals and nitric oxide are generated in surplus. Pathogenesis induced by free radicals is of great importance due to the evidence that these nitric oxide and oxygen radicals play a major role in the pathogenesis of many infectious diseases.

Reactive nitrogen oxide formed endogenously during viral replication possesses the ability to mutate viruses and host, leading to oxidative stress through oxidation and nitration of biomolecules(9). NDV velogenic and mesogenic strains are able to cause oxidative stress that increases malonaldehyde levels and decreases GSH, GPx, GR, and GST levels in the organs of infected chicken(10,11). Glutathione is a multifunctional tripeptide that is a powerful antioxidant and a key molecule in xenobiotic drug metabolism. Its maintenance is pivotal for cellular defense against oxidative injury(12). Protein nitration is considered as a final product of the oxide intermediates formed by nitric oxide and oxygen derivatives, such as superoxide. Increased RNS during viral replication leads to oxidative stress and damage to biological molecules including proteins, DNA, and lipids(13).

Antiviral drugs play a major role in controlling various viral infections such as Herpes Simplex Virus (HSV), Human Immunodeficiency Virus (HIV), Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Cytomegalovirus (CMV) infections. The most prominent antiviral drugs are nucleoside derivatives, which act as potent inhibitors of growing chain elongation, cellular, viral DNA and RNA polymerases, and reverse transcriptase's (RTs). All of these targets play an important role in the viral life cycle (14,15). Didanosine (DDI) (5'-O-2'-3'-dideoxydidanosine), a synthetic nucleoside purine analogue, was a second antiviral HIV drug approved by the FDA. Upon inactive distribution to the target cell, DDI undergoes phosphorylation by cellular enzymes resulting in the active moiety dideoxyadenosine-5 -triphosphate (ddATP). The active ddATP competes with natural deoxyadenosine 5 - triphosphate and cellular enzymes. Due to the lack of a 3 - hydroxyl group which is present on the natural substrate, incorporation of ddATP leads to chain termination of viral DNA and thus inhibits growth of virus(16). Didanosine has many disadvantages such as low plasma half-life (one hour), restricted penetration to the central nervous system, and low bioavailability (20-40%).As a result, Didanosine requires continuous infusion in order to maintain a therapeutic plasma level(15). The clinical use of Didanosine and other viral reverse transcriptase nucleoside inhibitors has had several severe side effects, such as the formation of resistant viruses(17). The development of prodrugs has gained significant attention in the field of modern medicine as a potential effective method of therapy. Prodrugs are modified chemical derivatives used in therapy because of their greater stability, higher permeability and increased solubility. These characteristics are primarily due to the physico-chemical properties in the inactive form that later become activated by biotransformation or chemical transformation. The biological effect is exhibited by the subsequent active derivatives. Benefits of using prodrugs include the reduction of unfavourable characteristics such as cytotoxicity, and an increase in bioavailability, time of action, and absorption. Prodrugs designed to deliver monophosphate nucleosides inside the cells have proven to be efficient in improving the therapeutic potential of antiviral and anticancer nucleosides. Once inside the cell, the monophosphate nucleoside is converted to its active form by phosphorylation(18). So far, there is no specific treatment available for NDV treatment. Antibiotics are recommended to prevent secondary infections (antibiotics don't affect viruses). Vaccination and sanitation measures are followed as a preventive measure(19). Due to the several advantages of prodrugs, a novel phosphorylated analogue for the Didanosine compound was designed, synthesized, characterized and tested against NDV infection in in vitro and in vivo studies of our previous paper.Previously we established in vitro and in vivo models of NDV infection and antiviral activity of novel phosphorylated analogue DDI-10 using a DF-1 chick embryo fibroblast cell lines and one day old chicken. Its efficacy in the reduction of viral load was also presented(20). Newcastle disease virus infection affects the poultry industry and the associated high mortality rate, with the virus induced pathogenesis caused by oxidative stress being inevitable during infection. Therefore, this study is focused on elevating the ameliorative effect by novel phosphorylated analogue DDI-10 in experimentally infected day-old chicken. Since viruses cause cell damage by generating oxidative stress leading to viral pathogenesis, the present study assesses the protective role of the Didanosine compound's phosphorylated analogue against oxidative stress caused by infection from NDV. This study was performed by estimating the enzymatic antioxidant restoration levels in infected chicken, the ability to reduce protein nitration and oxidation in chicken vital organs, and the protective role of DDI-10 conferred against NDV pathogenesis and oxidative stress.

## Materials and methods

### Experimental animal maintenance

*Gallus domesticus*; BV 300 strain was purchased from Balaji Hatcheries, Chittoor. Aged one day old chicken were maintained in isolated cages with *ad libitum* to food and water in 12:12 light/dark cycles during the experimental duration.

### Animal ethical declaration

Institutional Animal Ethical Committee approval for the use of chicken was obtained from Sri Venkateswara University, Tirupati. (No.14/2012-2013/(1)/a/CPCSEA/IAEC/SVU/VL-WR dt.23.01.2013).

### Virus

Komarov mesogenic Newcastle Disease Virus reference strain was received as a generous gift from Department of Microbiology, Sri Venkateswara Veterinary University, Tirupati, A.P. Infectious dose $ID_{50}$ of the virus was calculated to be $10^{9.4}$ units/mL(11).

### Experimental groups

Synthesis, characterization, and antiviral activity of novel phosphorylated DDI-10 and its requisites needed to carry out the current study was reported earlier in our previous publication(20). Briefly, one day aged chicken was brought to the animal house and kept for one day for the acclimatization period. Chicken were divided into three groups each with six individuals; Group-1 (healthy control): Three days old chicken were given 200µL of Phosphate Buffered Saline(PBS) and served as the healthy control; Group-2 (infection control): 200µL NDV via intramuscular route was given to three days old chicken (considered as day 0) and were monitored for clinical symptoms throughout the experimental period; Group-3 (DDI-10 treatment): 200 µL NDV was given intramuscularly starting at day 4 of post infection, in which 1.5 mg/kg body weight dose of DDI-10 treatment was administered orally every day for 10 days. On the $11^{th}$ day lung and liver tissues were isolated from all the groups; a piece of excised tissue was fixed in 10% formaldehyde for immuno histological studies. Tissues intended for estimation of antioxidant enzymes and western blot were stored at -40°C until further analysis.

### Assay of enzymatic antioxidants

### Assay of Glutathione Peroxidase (GPx) (EC 1.11.1.9)

The activity of glutathione peroxidase (GPx) was determined according to the method of Rotruck et al. (1973)(21). Tissues were blotted dry, thawed, and homogenate of lung and liver tissues (10% w/v) were prepared in 50mM phosphate buffer (pH 7.0) containing 0.1mM EDTA. The enzyme estimation from tissue homogenates was carried out immediately after homogenization. The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C. The ensuing supernatant was used as the enzyme source. The reaction mixture consisted of 500µl of phosphate buffer, 100µl of 0.01M GSH (reduced form), 100µl of 1.5mM NADPH, and 100µl of glutathione reductase (0.24 units). The 100µl of tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Next, 50µl of 12mM tetra-butyl hydroperoxide was added to 450µl of tissue reaction mixture and measured at 340 nm for 180 sec. The molar extinction coefficient of $6.22 \times 10^3$ M $cm^{-1}$ was used to determine the activity. One unit of activity is equal to the µ moles of NADPH oxidized / mg of protein / min.

### Assay of glutathione-S-transferase (GST) (EC 2.5.1.18)

Glutathione-S-transferase (GST) activity was estimated by the method of Habig et al. (1974) (22) using 1-chloro-2, 4 dinitrobenzene (CDNB). The enzyme source was prepared as described in GPx activity without any changes. The reaction mixture with a final volume of 2.9ml contained 1.0mM potassium phosphate buffer (pH 7.5), 1.0mM CDNB, 5.0mM GSH, and 0.1ml of enzyme source. The reaction was initiated by adding GSH at room temperature. The change in absorbance for one minute was recorded in a Hitachi U-2000 dual beam spectrophotometer. The enzyme activity was calculated using a molar extinction coefficient of $9.6 \times 10^{-3}$. The enzyme activity was expressed as µ moles of CDNB-GSH conjugate formed / mg protein / min.

### Estimation of Reduced Glutathione

Reduced glutathione content was estimated by the method of Sedlak and Lindsay (1968)(23). The lung and liver tissues were homogenized in 0.1M ice cold phosphate buffer (pH 7.0) containing 0.001M EDTA. Protein was precipitated with 1 ml of 5% sulfosalicylic acid (w/v) and the contents were centrifuged at 5000g for 15 min at 4°C. The resulting supernatant was used as the enzyme source. The reaction mixture with a total volume of 2.5ml contained 2.0ml of 0.1M potassium phosphate buffer, 0.005ml of NADPH (4 mg /ml of 0.5% $NaHCO_3$), 0.02ml of DNTB [5,5'-dithio-bis-(2-nitrobenzoic acid)] (1.5mg/ml), and 0.02ml of glutathione reductase (6 units/ml). The reaction was initiated by adding 0.41ml of enzyme source and change in absorbance was recorded at 425 nm against the reagent blank. The glutathione content was expressed in nanomoles/gram wet weight of the tissue.

### Protein oxidation by ELISA

Briefly, 10% w/v tissues of liver and lung from control, NDV infected and NDV + DDI-10 treated groups were taken and homogenized. 200 µL of 100 µg/mL homogenate of liver and lung tissue in PBS was added to a 96 well plate and incubated at 4°C overnight followed by three washes with PBS. PBS without protein was used as negative control. DNP solution of 200 µL was added at room temperature and allowed to sit for 45 minutes at dark. After incubation 1:1 V/V (PBS: ethanol) was used to wash. Next, 5% (200µL) skimmed milk powder was used in blocking for one and a half hours following a wash with PBS containing 0.1% tween 20 (PBS-T). Primary antibody rabbit anti-DNP (200µL; 1:500 diluted in blocking buffer) was added in each well for 1 hour and washed

with PBS-T. Secondary antibody anti-rabbit (200µL; 1:2500 diluted in blocking buffer) was added and incubated for 1 hour and washed with PBS-T. For 4-5 minutes 200µL of TMB substrate was added and 100µL of 2N HCl was used in stopping the reaction, which was subsequently read at an absorbance of 450 nm.(24)

### Protein nitration by ELISA

Triplicates of liver and lung tissue homogenate protein samples (100µg/100µL) from control, infected and treated groups were added to 96 well plates coated with nitrated BSA dissolved in 50 mM carbonate buffer (pH 9). Primary antibody rabbit anti-nitro tyrosine 100µL (1:200) dilutions were added, incubated for 120 minutes at room temperature, and washed with a wash buffer. Anti-rabbit secondary antibody conjugated with HRP (100µL diluted to 1:5000) was incubated for 60 minutes. TMB 100µL solution was used as a substrate and 2N $H_2SO_4$ 100µL was used for stopping the reaction, which was subsequently read at an absorbance of 492 nm.(25)

### Detection of protein oxidation and nitration by double immunofluorescence (Immuno co-localization)

Primary antibodies anti-DNP (Invitrogen, USA) and anti-nitro tyrosine (Cayman, USA) are marker antibodies commonly used to measure protein oxidation and nitration. In this study these antibodies were utilized to analyze the extent of NDV induced oxidative damage in the vital liver and lung tissues of chicken in control, NDV infected and NDV infection+DDI-10 treated groups. Sections were sequentially dipped in xylene, ethanol, and distilled water to deparaffinize tissues. Derivatization with 0.1% DNPH in 2 N HCl was carried out to measure protein oxidation levels following a 4°C overnight incubation. The derivatization step was excluded for the protein nitration. Quenching of tissue was completed using 0.3% $H_2O_2$ for half an hour, followed by three PBS washes and blocking of the tissues with 10% normal serum in PBS for 30 minutes. Primary antibody rabbit anti-DNP in 1:100 dilutions and primary antibody rabbit anti-nitro tyrosine in 1:200 dilutions used to detect oxidation and nitration respectively were coated on tissue sections incubated overnight at 4°C, which were immediately followed by three PBS washes. Alexa fluor 488 (Invitrogen, USA) in dilutions of 1:500 with an anti-rabbit secondary antibody was added onto the tissue sections and allowed to stand for 1 hour at dark. Additional primary antibodies of mouse anti α-SMA (1:400; liver: Abcam, USA) and mouse anti-clara (1:500; lung: Invitrogen, USA) were used to label the hepatic stellate cells and clara cells for 60 minutes at room temperature. This was followed by washes with PBS and a second secondary antibody anti-mouse Alexa fluor 594 (Invitrogen, USA) in dilutions of 1:500 for 1 hour. Sections were then washed with PBS and an anti-profade reagent was added(26). Images were captured at three random fields in an Olympus BX-51 model fluorescence microscope. Images were analysed for co-localised protein fluorescence intensity with Image J Software.

### Western blot analysis

Protein oxidation and nitration analysis of liver and lung tissues was carried out using antibodies anti-DNP and anti-nitrotyrosine from the healthy control group, NDV infected group, and NDV infection+DDI-10 treatment group tissues. Protein from lung and liver tissues were extracted in the RIPA buffer (10% w/v). The proteins were derivatized with 20mM DNP (10µL) at room temperature for 15 minutes, which was followed by the addition of 5µL of neutralization solution to carry out protein oxidation. The 30µg protein sample was then loaded on to 10% SDS-PAGE and electro blotting was performed for 45 minutes at 55 volts using a 0.2µm PVDF membrane following an immediate wash with TBS. Next, 2% BSA in TBST was used as a blocking agent for 120 minutes, which was followed by three 10-minute washes with TBST. Rabbit anti-DNP primary antibody (1:1000) was added and incubated overnight at 4°C. After a wash with TBST, goat anti-rabbit IgG (1:5000) horseradish peroxidase conjugate was added and allowed to stand for 2 hours at room temperature. This was followed by three 10-minute washes with TBST. The protein bands developed on the membrane were visualized using the chemiluminescence substrate method. For the protein nitration 3-nitrotyrosine rabbit monoclonal antibody (1:1000) was used as the primary antibody and immunoblotting was performed with the same experimental conditions, excluding the protein derivation step that followed in the protein oxidation (27,28).

## Results

### Disease progression

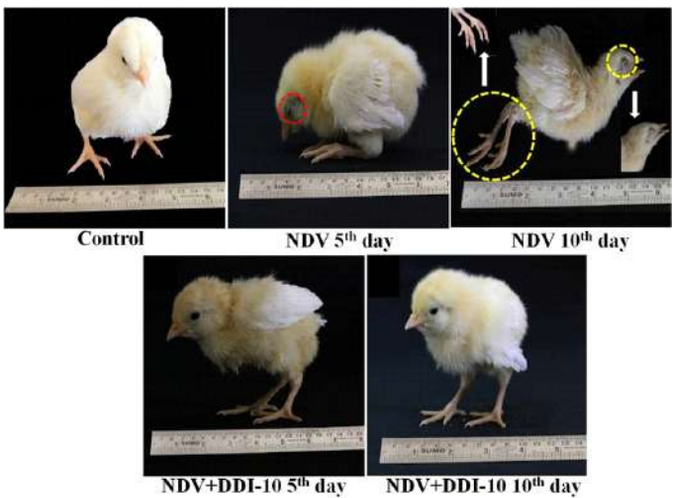
Three days old chicken were infected with NDV and administered with DDI-10 as described in the experimental section to assess DDI-10 antiviral efficacy and disease pathogenesis. All animals were monitored for clinical symptoms and signs of disease (i.e., nasal discharges, ruffled feathers, watery diarrhea, twisted neck and paralyzed legs) during the experimental period. NDV induced pathological manifestations and disease progression in infected chicken exhibited ruffled feathers and swelling of the tissue around the eye on the second day of virus infection and paralyzed legs, greenish diarrhea and heavy breathing on the fifth day of virus infection. Severe paralysis, twisted neck, and nasal discharges were noted on the $10^{th}$ day of NDV infection. Control chicken was void of any disease symptoms. All disease symptoms in NDV infected chicken were noticeably reduced upon treatment with novel phosphorylated compound DDI-10 (Fig. 1).

### Antioxidant Enzyme Activities

Glutathione is a potent antioxidant with high redox potential and serves as a cofactor for several oxidative stress detoxifying enzymes (glutathione peroxidase and glutathione transferase). The depletion of GSH levels characterizes viral infections and associated-disease progression. Therefore, antioxidant enzyme levels were crucial in evaluating the liver and lung tissues. Lung and liver

**Fig. 1**

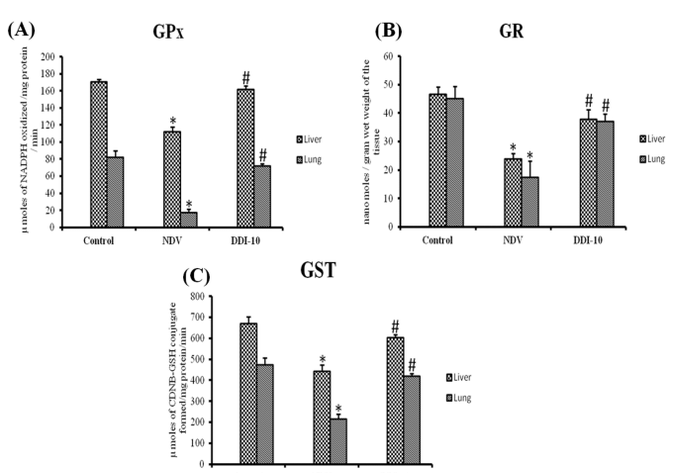
**Newcastle disease virus induced pathological manifestations in chicken**: *NDV infected chicken displayed clinical symptoms throughout the experimental duration.*




tissues were collected from NDV infected and NDV+DDI-10 treated chicken on day 13, GPx, GST and GR levels were analysed. The results revealed a significant ($p<0.05$) decrease in GPx, GST and GR in liver tissue (112.2 ± 5.49; 444.30±27.81; 23.97 ± 1.79) and lung tissue (17.63 ± 3.47; 216.37±23.08; 17.55 ± 5.63) of NDV infected chicken (Fig. 2 A, B and C) when compared with control group chicken (liver 170.29 ± 3.45; 669.73 ± 33.74; 46.58±2.61: lung 82.04 ± 7.57; 475.14 ± 31.31; 45.05±4.28). However, these perturbations significantly ($p<0.05$) recovered to normal condition in the DDI-10 treated group liver (161.89 ± 3.74; 603.99±13.29; 37.84 ± 3.42) and lung (71.97 ± 2.36; 420.73±10.63; 37.11 ± 2.51).

**Fig. 2**

***Effect of DDI-10 on antioxidant enzymes in control, NDV infected and DDI-10 treated chicken***: *Enzymes (A) GPx, (B) GR, and (C) GST were estimated from the liver and lung tissue of chicken from all the groups. The enzyme activity levels of the DDI-10 treated group regained significantly. Hence, phosphorylated compound DDI-10 significantly increased the enzyme activity levels (n=6). Enzyme estimation was done in triplicates. *Comparison between control and NDV groups ($p<0.05$). #Comparison between NDV and DDI-10 groups ($p<0.05$).*

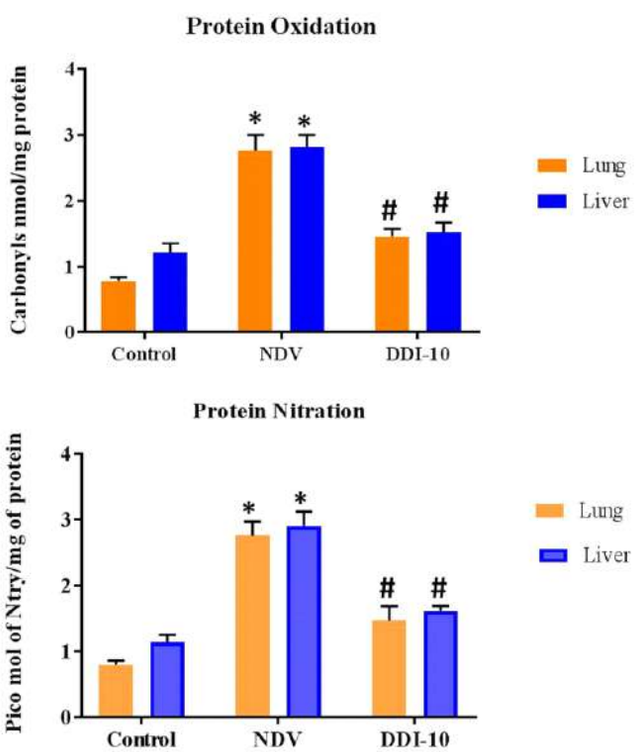



**Protein oxidation by ELISA**

Protein oxidation is the covalent modification of proteins either directly by ROS or indirectly by its reactive secondary metabolites. These oxidative modifications are induced in various diseased conditions *in vivo*; protein oxidation serves as a marker in measuring the oxidative stress in vivo. Hence, protein oxidation levels in liver and lung tissue during NDV infection were measured in control, NDV infected, and DDI-10 treatment groups using antibody anti-DNP by ELISA. Protein was extracted from the respective tissue collected from each group on day 13 in the RIPA buffer and using homogenate protein oxidation was estimated by ELISA. In comparison with control chicken, the protein oxidation levels were significantly ($p<0.05$) increased in the NDV infection group. An increased protein oxidation level was brought down significantly upon treatment with NDV+DDI-10 in the treated group compared to the infected control group (Fig. 3).

**Fig. 3**

***The content of protein carbonyls and nitrated protein were measured in control, NDV infected, and NDV+DDI-10 groups by ELISA in lung and liver tissues***. *Protein was extracted from infected and treated animals and analysed for protein oxidation as content of carbonyls and nitration as nitrated protein levels. Values presented are from triplicates with mean and SD (n=6). *Between control group and NDV-infected group ($p<0.05$). #Between NDV infected and treatment groups ($p<0.05$).*




**Protein nitration by ELISA**

Peroxynitrite radicals are an imperative reason for the type of oxidative damage known as protein nitration and have a significant role during disease pathogenesis. Therefore, protein nitration levels in liver and lung tissue of

control, NDV infection, and NDV+DDI-10 treated groups were measured by ELISA. Tissues collected from chicken on day 13 were homogenized in RIPA buffer and used to detect nitrosylated protein using 3-nitrotyrosine antibody. In comparison to the healthy control chicken, protein nitration levels were significantly increased in the NDV infection group. Upon treatment with DDI-10, the raised levels of nitrated protein were significantly decreased in comparison to the diseased alone group (Fig. 3).

**Detection of protein oxidation and nitration by double immunofluorescence (Immuno co-localization) and Western blot assay**

Since ELISA results demonstrated oxidative damage during NDV infection indicating viral pathogenesis, and subsequently decreased oxidative damage upon treatment with DDI-10, further quantification assays such as immuno co-localization and western blot analysis were carried out. Immuno co-localization studies were performed in lung and liver tissues from control, NDV infected, and NDV+DDI-10 treated group chickens. Anti-nitro tyrosine and anti-DNP are used to analyse nitrosylation and oxidation of proteins. Stellate cell marker used in liver tissues and clara in lung tissues to localize the oxidative stress in the respective tissues. Analyzed immuno co-localization sections revealed that NDV infected chickens have more oxidized protein with a large number of hepatic stellate cells in liver tissue(Fig. 4 H) and clara cells in lung tissue (Fig. 5 H) in comparison tohealthy control animals (Fig. 4 & 5 G). In contrast, cellular alterations were either minimal or decreased in the liver and lung tissue of the NDV infected group treated with NDV+DDI-10 (Fig. 4 & 5 I). Immunofluorescence studies of protein nitration were carried out with an anti-nitrotyrosine antibody. Similar to the protein oxidation, protein nitration was also significantly altered in the NDV infected animal tissues (Fig. 5 & 6 H) whencompared to control animals (Fig. 6 & 7 G). These alterations were decreased significantly in theNDV+DDI-10 treated group (Fig. 6 & 7 I). Protein nitration and oxidation were also measured using the western blot method. Tissue samples from the control, NDV infected, and NDV+DDI-10 treated groups were subjected to blotting using anti-DNP and 3-nitrotyrosine antibodies for protein oxidation and nitration, respectively. Blotting revealed a distinct band pattern for each group. Bands were noticeably more intense in the NDV group compared to the control group, indicating the presence of a large amount of oxidation and nitration. These alterations in the protein oxidation and nitration levels were brought down with the treatment of DDI-10 (Fig. 8).

**Fig. 4 (I)**

***Double immunofluorescence-stained sections of liver tissue with Anti-DNP and α-SMA were used to estimate oxidized protein****. Immunofluorescence of Anti-DNP (A,B,C), α-SMA (D, E, F), and merged images (G, H, I) were captured from liver tissues of experimental and control animals. A greater amount of immuno co-localized cells (H) in liver tissue was noticed compared to healthy control and DDI-10 treated birds. Scale bar (----------) = 50 µm.*

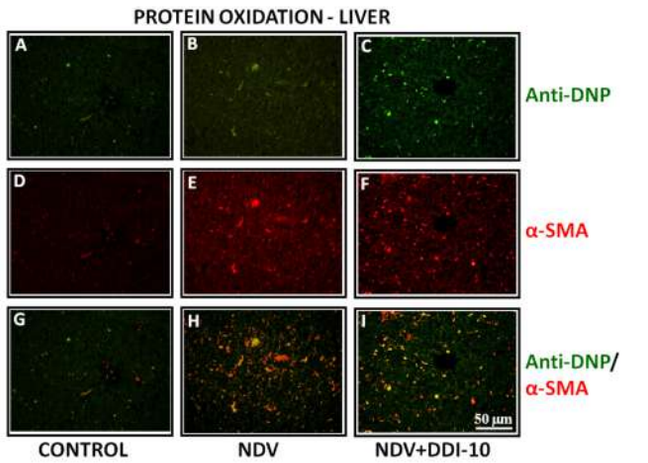



**Fig. 4 (ii)**

*The fluorescence density quantification of immuno co-localized sections from G,H& I.*

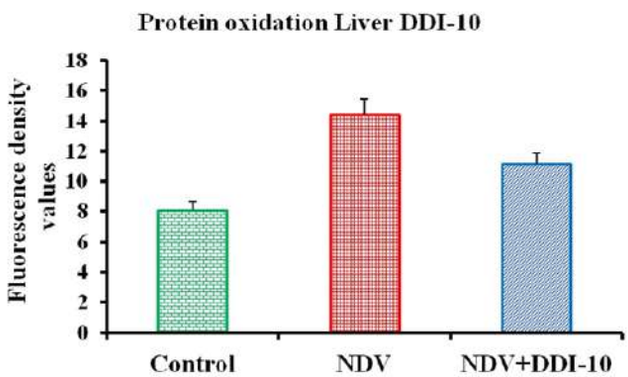



**Fig. 5 *(I)***

***Double immunofluorescence-stained sections of lung tissue with Anti-DNP and anti-clara primary antibodies****. A,B,C-Anti-DNP, D,E,F-anti-clara, and G,H,I- colocalized. A considerable amount of immuno co-localized cells (oxidized protein) in lung tissue was observed (H) when compared to control and DDI-10 treated birds. Scale bar (----------) = 50 µm.*

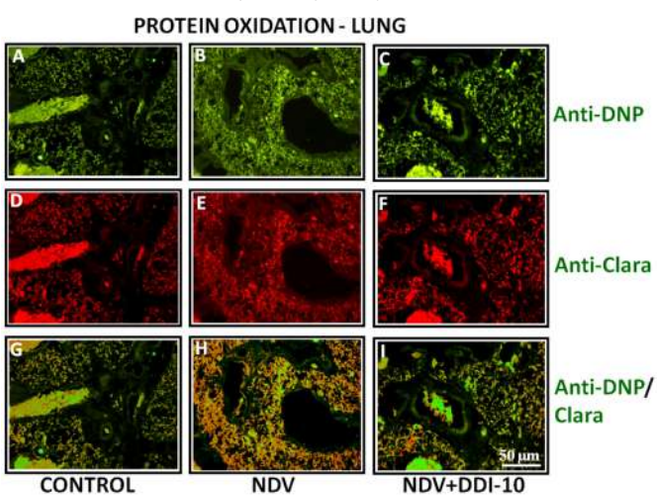


**Fig. 5 (ii)**

***The fluorescence density quantification of immuno co-localized sections from G,H& I.***

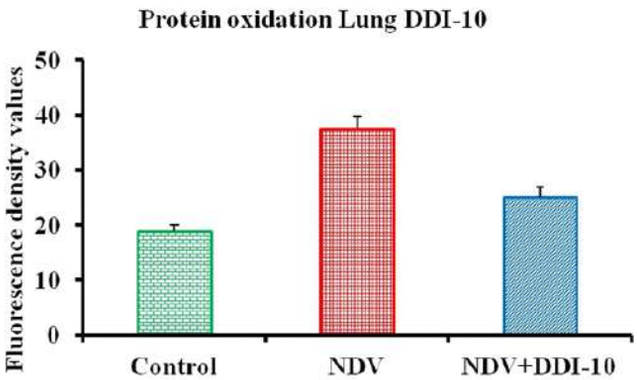



**Fig. 6 (I)**

***Double immunofluorescence photomicrograph sections of liver tissues stained with Anti nitro tyrosine and α-SMA****. A,B,C-Anti-nitro tyrosine, D,E,F- α-SMA, and G,H,I- colocalized. H-Showed a large amount of nitrated protein due to NDV infection, whereas DDI-10 treated showed a lesser number of colocalized cells (I) when compared to control tissue (G). Scale bar (----------) = 50 µm.*

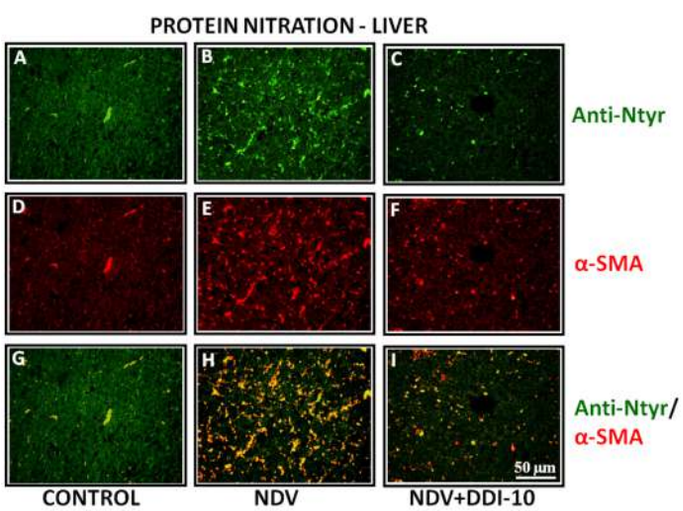



**Fig. 6 (ii)**

***The fluorescence density quantification of immuno co-localized sections from G,H& I.***

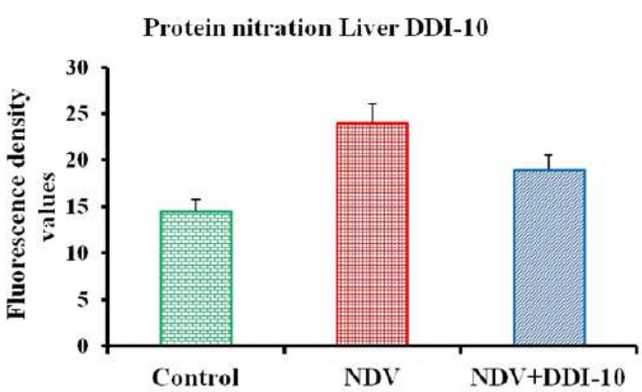



**Fig. 7 (I)**

***Double immunofluorescence photomicrograph sections of lung tissues stained with Anti-nitro tyrosine and anti-clara primary antibodies****. A,B,C-Anti-nitro tyrosine, D,E,F-anti-clara, and G,H,I-colocalized. H-Tissue from NDV infected animals showed a greater number of colocalized cells than that of (I) DDI-10 treated birds. G-Control birds showed only a few nitrated cells. Scale bar (--------) = 50 µm.*

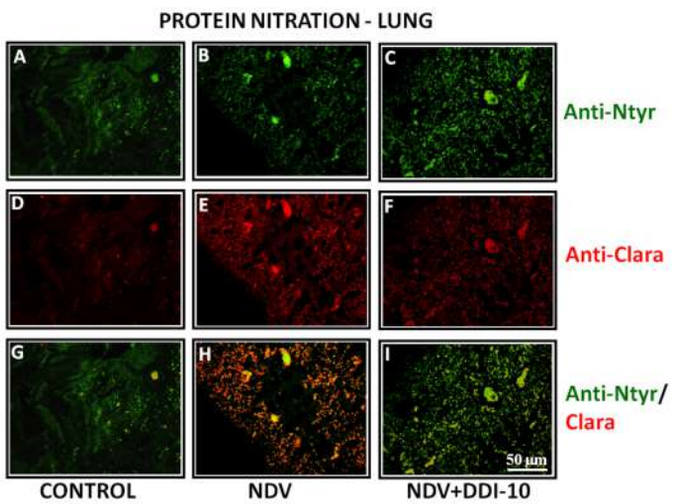
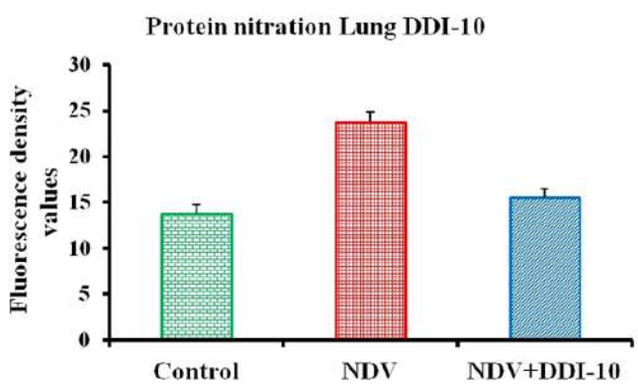



**Fig. 7 (ii)**

***The fluorescence density quantification of immuno co-localized sections from G,H& I.***

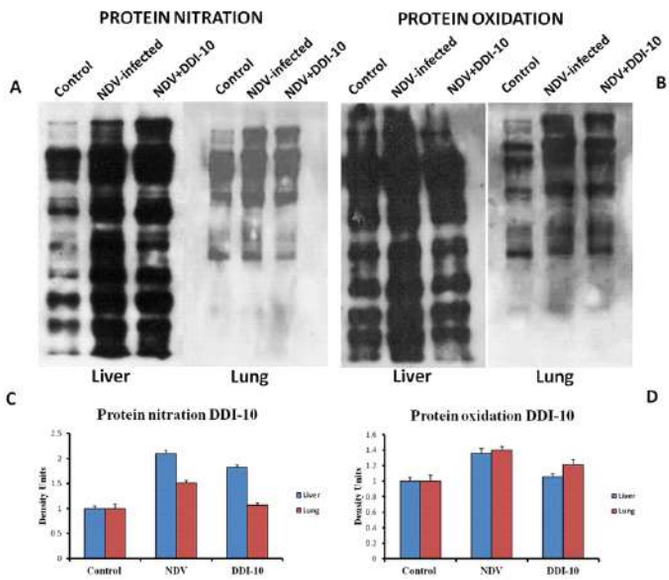



## Discussion

In spite of vast research being performed for NDV influenced damage to the poultry industries; very few studies addressed role of oxidative stress in poultry(29). Many RNA viruses are able to induce oxidative stress that leads to an increase in viral replication and cell death (10). Oxidative stress is implicated as a pathogenic factor in a number of viral infections. It is evident that most of the infections cause production of ROS (reactive oxygen species) and RNS (reactive nitrogen species), which is predominant for infections such as hepatitis viruses, human immunodeficiency virus (HIV), influenza A, Epstein-Barr virus, respiratory syncytial virus and many other viruses(30). Pathogenicity induced by free radicals produced during oxidative stress of viral infections is of vast importance due to its key role in the pathogenesis of various infectious diseases. Although production of oxygen radicals and nitric oxide radicals has an

**Fig. 8**

***(i) Protein nitration and oxidation in control, NDV infected, and NDV+DDI-10 treated chicken were measured by western blot analysis.*** *(A) Nitrosylation of proteins was detected on the blot using an anti-nitro tyrosine antibody. (B) Oxidation of proteins were detected on the blot using an anti-DNP antibody. Relative density was calculated from three individual blots by Image J and plotted as (C and D). Increase in band intensity was observed in the NDV infected group animals when compared to control; in the treatment group DDI-10 band intensity was reduced.*




antimicrobial effect on bacteria and protozoa, these radicals have contrasting effects in viral infections such as influenza and neurotropic disease (9).

Although ROS and RNS have a prominent role in several antimicrobial and antitumor activities, they are also involved in pathogenesis for a wide range of diseases(31). Due to its role in cell activation, ROS may facilitate or promote replication of these parasites based on the virus and cells involved(32,33). Dysregulation in cellular metabolism leads to oxidative stress, resulting in higher levels of ROS and RNS production subsequently damaging the tissue and creating a disturbance in the antioxidant enzyme levels(34). The detrimental effect of oxidative stress is eliminated in the body by enzymatic and non-enzymatic antioxidants. Oxidative stress may be caused by increased ROS generation and/or a depressed antioxidant system. The natural antioxidant system consists of numerous antioxidant compounds and several antioxidant enzymes such as GPx, SOD and CAT. The primary ROS produced in aerobic organisms is a superoxide radical that is also a highly reactive cytotoxic agent. Superoxide radicals are converted to $H_2O_2$ by SOD. $H_2O_2$, in turn, is converted to molecular oxygen and $H_2O$ by either CAT or GPx. Additionally, GPX can reduce lipid peroxides and other organic hydroperoxides that are highly cytotoxic products. Therefore, SOD, CAT and GPX constitute the principal components of the antioxidant system and their deficiencies can cause oxidative stress(35).

Glutathione is a multifunctional tripeptide that is a powerful antioxidant and a key molecule in the xenobiotic drug metabolism. Its maintenance is pivotal for cellular defense against oxidative injury and preservation of cellular integrity(12). Thus, alterations in the glutathione metabolism may affect the glutathione dependent enzymes subsequently leading to poor detoxification mechanisms(36). In the present study, selected vital organs of NDV infected lung and liver noticed a significant decrease in the levels of glutathione (GPx, GR and GST) dependent enzymes. This decrease in the activity levels of these enzymes suggests improper scavenging activity of free radicals during viral infection. The fact that these enzyme levels were recovered to normal state in the treatment group of DDI-10 indicate a protective role against NDV induced oxidative damage pathogenesis. Studies involving vitamin E treatment against NDV in our laboratory have shown a similar type of results regarding the glutathione enzyme status; in a non-treated group enzyme levels were significantly decreased, while a significant increase was observed in the treated group(11). A study by Rehman et al. 2018(10) stated glutathione levels were decreased in NDV infection and recovered with Vitamin E treatment. Hence, the results obtained in the present study are well supported by earlier findings.

Cellular proteins are prone to oxidative stress in the presence of various ROS; depending on the ROS, the damage made to proteins may be of nitration and/or oxidation of amino acid groups in proteins which are stable markers for detection of oxidative damage. Protein nitration is known to be the end product in the RNS intermediate form(37). In the current study, oxidative stress induced pathogenesis in experimentally infected chicken with NDV was measured by 2, 4-dinitrophenyl hydrazine (DNPH) and 3-Nitrotyrosine primary antibodies which were specific for detecting protein oxidation and nitration using ELISA, Western and immuno co-localization studies. ELISA tests were carried out to estimate the damage of oxidative stress in the infected group of chicken. Compared to the control group, a clear positive for protein carbonyls and nitrated proteins was demonstrated in the infected group (Figure 3). In addition, the increase observed in the infected group was brought down in the treatment group with DDI-10 (Fig. 3). To further confirm and evaluate pathogenesis of oxidative stress within lung and liver tissue, western blot analysis and immuno co-localization assays were performed in the control, NDV infected, and DDI-10 treated groups. Western blots and immuno co-localization assays were in correlation with ELISA results, in which the western blot band pattern in the infected group tissues and the associated immuno co-localization sections revealed a greater amount of co-localized cells in the NDV alone group compared to the healthy control group chicken. These perturbations were reduced during the treatment with novel phosphorylated analogue DDI-10. Previous studies in our laboratory with NDV infected chicken have shown increased levels in protein oxidation and nitration, which were subsequently brought down with the supplementation of vitamin E(13). Based on the studies of Rehman et al. 2018,(10) NDV infection in chicken leads to oxidative damage in organs such as the jejunum and duodenum; supplementation of

Vitamin E altered these changes, minimized virus load, and ultimately helped these animals recover from the disease. It is clear from past research that many of these viruses cause protein oxidation and nitration due to oxidative damage. Antiviral compounds that were intended to provide protection from these viral infections consequently produced oxidative damage. P-icariin, a phosphorylated compound, has provided protection against oxidative damage and improved the survival of animals from the Duck viral hepatitis infection(38).

## Conclusion

In conclusion, NDV infection of chicken induces oxidative stress in the vital organs of the liver and lungs, subsequently mediating pathogenesis. Enzymatic antioxidants and glutathione dependent enzymes were significantly reduced in the NDV infected group, whereas these levels were increased in the DDI-10 treated group. Protein oxidation and nitration levels were significantly raised in the NDV infection group. The elevated levels were decreased in the novel phosphorylated DDI-10 treated group. The results demonstrated in this study suggest that NDV induced pathogenesis due to oxidative stress can be overcome by treatment with novel phosphorylated prodrug DDI-10. Therefore, it is summarized that this novel phosphorylated compound mitigates the NDV induced pathogenesis and protects chicken from NDV. Hence, prodrug DDI-10 has great potential to serve as a potent antiviral compound against NDV.

## Conflict of interest

The authors declare no conflict of interest.

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