Article

Newcastle Disease Virus induced pathologies severely affect the exocrine and endocrine functions of the pancreas in chickens

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Abstract:

Newcastle disease virus (NDV) causes a highly contagious and devastating disease in poultry, Newcastle disease (ND). ND causes heavy economic losses to the global poultry industry by decreasing the growth rate, decrease egg productions, mortality, and morbidity. Although, significant advances have been made in the vaccine development, but outbreaks are reported in vaccinated birds leading to overall decreased growth rate. In this study, we report the damage caused by the NDV infection in the pancreatic tissues of vaccinated as well as specific pathogen free chickens. The histopathological examination of the pancreas showed sever damage in the form of partial depletion of zymogen granules, acinar cell vacuolization, necrosis, and apoptosis, congestion in the large and small vessels, sloughing of epithelial cells of pancreatic duct, and mild perivascular edema. Increased plasma levels of corticosterone, somatostatin, were observed in NDV infected chicks at 3 and 5-day post infection (DPI). Slight decrease in the plasma concentrations of the insulin were noticed at 5 DPI. Significant changes were not observed in the plasma levels of glucagon. Furthermore, NDV infection has decreases the activity and mRNA expression of amylase, lipase, and trypsin from the pancreas. Taken together, our findings highlight that NDV induces extensive tissue damage in pancreas, decrease the activity and expression of pancreatic enzymes and increase plasma corticosterone and somatostatin.

Keywords: Newcastle disease virus; chicken; pancreas; hormone; enzymes; histopathology

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1. Introduction

Newcastle disease (ND) is highly infectious and one of the most distressing viral diseases of poultry, leading to the heavy economic losses to the global poultry industry [1]. ND is caused by the virulent strains of Avian orthoavulavirus 1 (AOAV-1), also known as ND virus (NDV) [2]. AOAV-1 belongs to the order Mononegavirales, family Paramyxoviridae, and genus orthoavulavirus, and contains non-segmented, nega-

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tive-sense, and single stranded RNA genome of 15kb length [3]. The proteins encoded by the AOAV-1 genome are fusion (F), haemagglutinin-neuraminidase (HN), matrix (M), phosphoprotein (P), and large polymerase (L). From these proteins, F and HN are mainly involved in the AOAV-1 induce pathologies in poultry.

There are four pathotypes of AOAV-1; asymptomatic enteric, lentogenic, mesogenic, and velogenic [4]. Mesogenic and velogenic strains of AOAV-1 are responsible for the systemic infections in poultry, and clinical manifestations of variable mortality from subclinical infections to 100 percentages. ND mainly affect the digestive, respiratory and nervous system [5-8], but not limited to these organs. AOAV-1 induced pathologies and replication had been noted in spleen, liver, bursa of Fabricious, heart, reproductive system [9-13]. Previous outbreaks of AOAV-1 in vaccinated as well as non-vaccinated poultry had reported the involvement of pancreas [12,14,15].

For the digestion of lipids, proteins, carbohydrates, and nucleic acids; different enzymes are produced by the digestive system including salivary glands, intestine, liver, and pancreas [17]. Pancreatic enzymes such as trypsinogen, chymotrypsinogen, elastase and procarboxypeptidases for the digestion of proteins; pancreatic amylase for the digestion of carbohydrates; phospholipase, lipase, and cholesterol esterase for the digestion of lipids; and two type of nucleolytic enzymes namely ribonuclease and deoxyribonuclease [17-20]. The endocrine pancreatic tissue have clusters of cells known as islets of Langerhans, which produce different hormones including insulin, glucagon, somatostatin , and avian pancreatic polypeptide (APP) [21]. These hormones regulate the glucose homeostasis, glucose and amino acid transport, lipogenesis, glycogenolysis, hypoglycerolemia, regulate the level of plasma free fatty acid, regulate the expression of lipogenic enzymes, intestinal motility, and gall bladder secretions [21].

Previous studies have shown the involvement of pancreas in NDV infection and histopathological lesions were reported in chickens [12,14,22,23], ducks [2,24], geese [25], and turkeys [26]. However, the endocrine and exocrine functions of the pancreas are not clear in NDV infected chickens. Therefore, present study was planned to investigate the pathophysiology of pancreatic functions in NDV infected specific-pathogen-free (SPF) and vaccinated and then NDV challenged chickens.

2. Materials and Methods

2.1. Virus

A wild type velogenic NDV isolate, ZJ1, (Goose/China/ZJ1/2000; GB AF431744.3) was provided by Professor Xiufan Liu from Yangzhou University (Yangzhou, China). The pathogenicity indices including mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) of the ZJ1 were 51.6, 1.89, and 2.7, respectively [27,28]. The virus stock was prepared by growing the virus in 10-day-old SPF embryonated chicken eggs and harvested allantoic fluid was subsequently stored at −80 °C until further use.

2.2. Chickens and experimental design

Specific pathogen free embryonated eggs were purchased from the Merial Vital Laboratory Animal Technology Company (Beijing, China) and were incubated at the laboratory facility of SHVRI, CAAS. The hatched-out SPF chicks were reared, vaccinated, and challenged in positive pressure isolators in high containment facility. All the birds were provided with ad libitum access to feed and water throughout the experiment. At the age of 9 weeks, chickens were randomly divided into three groups having 20 chicken each.

Birds in the group 3 (VAC+CHA = vaccinated challenged) were vaccinated at the age of 9 weeks with 100 µl of La Sota at the dose rate of 10^3 embryo infective dose (EID50/0.1ml) via intranasal and eye drop routs, prepared in phosphate buffered saline (PBS). After 2 week of vaccination of VAC+CHA group, all the birds in group 2 (CHA =

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non-vaccinated challenged), and VAC+CHA group were challenged with ZJ1 suspended in ice cold PBS have a titer of $10^{6.5}$ EID50/0.1 ml per bird via right eye and choanal slit in 100 µl volume. Dose of the challenged and vaccinated viruses were selected on the basis of previous experiment of Cornax, *et al.* [27]. Birds in the group 1 (CON = control) were given the same dose of PBS, via the same routs, as a mock infection.

2.3. Sampling

At 1-, 3-, 5-days post infection (DPI), blood (in EDTA-coated tubes) and tissue (pancrease) samples were collected from four birds per group, and immediately transferred to the laboratory, maintaining the cold chain. To obtain the plasma, blood samples were centrifuged (2000× g) for 10 min at 4 ºC and stored at −80 ºC until further analysis. All the tissue samples were immediately put in liquid nitrogen and stored at -80 ºC until further analysis.

2.4. Plasma hormones

Plasma levels of the somatostatin, insulin and glucagon were determined by using chicken specific commercial assay kits [Nanjing Jiancheng Bioengineering Institute (HO92-SS; H183 GC; H203 Insulin)]. All samples were analyzed within single assay to avoid inter-assay variations. The absorbance of the microplates was determined using the Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) at the optical density value of 450 nm. The plasma levels of corticosterone (CORT) were measured using a chicken specific commercial ELISA kit (CSB-E11991C; Cusabio Biotech. Co., Ltd., Wuhan, China). The standard curve was created by using the CurveExpert 1.4. The limits of detection for the insulin and glucagon were 0.5mIU/ml and 5ng/L, respectively. The inter and intra assay coefficient of variation was less than 10 % and 12 % respectively.

2.5. Digestive enzyme activities in pancreas

To determine the activity of the pancreatic enzymes, a small portion (approximately 1 mg) of the pancreatic tissue was homogenized in 9 volumes (W/V) of ice-cold phosphate buffer saline (PBS), using a Tissuelyser-24 (Jingxin Technology, Shanghai, China) at 20 Hz for 2 minutes. The homogenate was instantly centrifuged at 3000× g at 4 °C for 10 minutes and the supernatant was collected for the determination of digestive enzyme activities. To determine the activity of trypsin (A080), lipase (A054) and amylase (C016) by calorimetric methods, commercial kits were used (Nanjing Jiancheng Bioengineering Institute, China). Pre-experimental standardizations were performed for each of the targeted enzyme to determine the optimum dilution for the measurement of enzymes. Protein concentration of the supernatants were determined by using the kits from Beyotime, (Beyotime, Nanjing, China; P0010).

2.6. Quantitative real-time PCR

The quantitative real time PCR (qRT-PCR) was performed to measure the mRNA expressions of pancreatic digestive enzymes including amylase, lipase, and trypsin genes. The chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. The primers used in the present study are described in the Table 1. Total RNA of the pancreas samples were extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and resuspended in RNase-free water. Reverse transcription was performed using HiScript II (Catalogue # R233; Vazyme Biotech Co., Ltd., China), according to the manufacturer's instructions. Expression of target genes were measured by performing the qRT-PCR on CFX96 Touch Real-Time PCR Detection System (BioRad, USA), using SYBR Premix (Dongsheng, Biotech, China), and data were normalized with GAPDH (internal control). The qRT-PCR was performed with a final volume of 20 µl. The PCR cycles are as follows: 94 ºC for 3 minutes, followed by 40 cycles of 95 ºC for 15 s, 60 ºC for 15 s and 72 ºC for 20 s. All the amplifications generated expected amplicons with

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single, sharp fusion curves. All experiments were carried out in triplicate. The changes of mRNAs were presented as fold expression and calculated using 2−ΔΔCT method [28].

2.7. Viral loads in the pancreas

The virus copy numbers were detected from the pancreas as described previously [9]. Briefly, the ZJ1 strain was grown in 10-day-old SPF embryonated eggs and allantoic fluid was collected after 60 h of infection. The TRIzol (Invitrogen, Carlsbad, CA, USA) reagent was used to extract the viral RNA from the allantoic fluid as per the manufacturer's instructions. The ZJ1 M gene was amplified using the primers mentioned in the Table 1, and confirmand the size (1095 bp) of the fragment by electrophoresis. The HiPure Gel Pure DNA Mini Kit (AnGen Biotech, Guangzhou, China) was used to purify the PCR product and cloned into a plasmid vector to construct a standard curve. The pancreatic tissue from the chickens was homogenized and RNA was extracted as mentioned above. About 1 μg RNA extracted from the pancreas samples was reverse transcribed to cDNA with HiScript II (Catalogue # R233; Vazyme Biotech Co., Ltd., China), and quantitative PCR was performed with SYBR Premix (Dongsheng, Biotech, China). Virus copy numbers were calculated using the standard curve [9].

2.8. Histopathology

Samples of the pancreatic tissue were collected from the middle region and fixed in 10% neutral buffered formalin, embedded in paraffin. and 5 μm thick sections were obtained. The sections were stained with hematoxylin and eosin (H&E) following the standard procedures [29]. The slides were digitalized by using Pannoramic SCAN (3DHISTECH Ltd., Hungary) and histological lesions were studied by using CaseViewer 2.2 (3DHISTECH Ltd., Hungary) [30].

2.9. TUNEL assay

For the detection of apoptotic cells, 5 mm thick tissue sections of the pancreatic tissue were subjected to the transferase dUTP nick-end labeling (TUNEL) assay according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Tissue sections were deparaffinized and rehydrated by the graded levels of xylene and ethanol. Tissue sections were treated with proteinase K and incubated at 37 ºC for 25 min. Activity of the endogenous peroxidase activity was blocked with hydrogen peroxide. The sections were incubated at 37 ºC with the terminal TdT nucleotide mixture for 2 hours. Nuclei were counterstained with 3 μg/ml 4′, 6′-diamidine-2-phenylindole (DAPI) and visualized by using fluorescence microscopy. Then slides were digitalized by using Pannoramic SCAN (3DHISTECH Ltd., Hungary), and studied by using CaseViewer 2.2 (3DHISTECH Ltd., Hungary). Five different high power fields (HPF) were selected to count the number of apoptotic cells using Image-Pro Plus software (version 6.0 for Windows).

2.10. Statistical analysis

The data were analyzed by two-way ANOVA test, with challenge and time points as the main effects [31].The graphical results were expressed as mean ± standard deviation (M ± SD). Results with $P < 0.05$ were considered as statistically significant. When a significant main effects was observed, TUKEY test was used to compare the differences among groups. Graph Pad Prism 6.0 software (GraphPad Software, Inc., CA, USA) was used to generate the graphs.

3. Results

3.1. Viral load in the pancreas

The transcriptional level of the NDV M gene was determined to examine the viral load in the pancreas using the qRT-PCR assay at 3 and 5 DPI. The viral copy numbers

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per µl are shown in the Figure 1. There was non-significant difference in the viral load of VAC+NDV and CHA groups at 3 DPI. The viral copy numbers were significantly less at 3DPI compared to 5 DPI in CHA and VAC+CHA groups. Maximum viral load observed in the CHA group at 5 DPI and was significantly higher compared to VAC+NDV group. The negative control group remained negative for NDV.

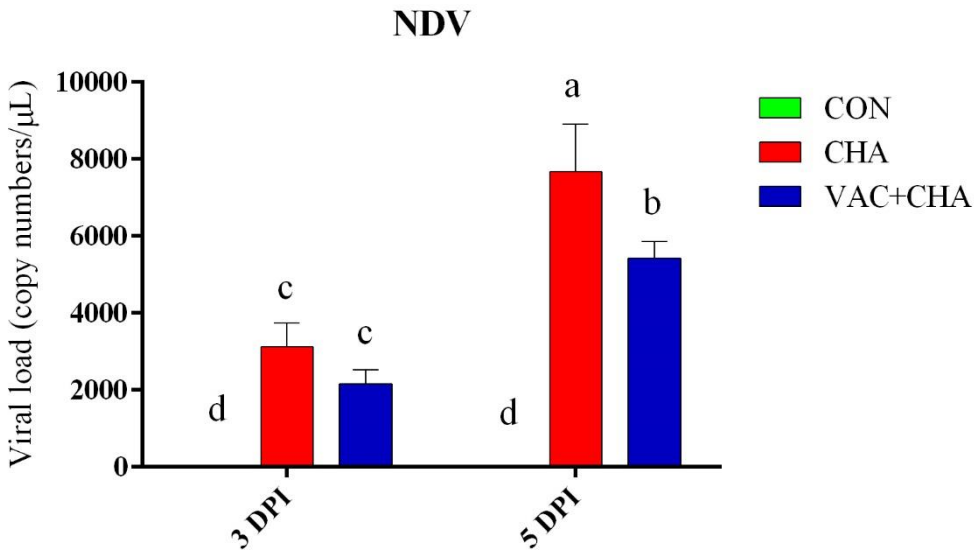

Figure 1. Relative quantity of Viral RNA in the pancreas of chicken. Graph is denoted as mean ± standard deviation. Columns without a common superscript letter differ significantly ($P < 0.05$). CON, Control; CHA, Newcastle disease virus challenged; VAC+CHA, vaccinated with LaSota and challenged with virulent Newcastle diseases virus after 2 weeks of vaccination.is a figure.

3.2. Histopathology

There were not significant changes observed in the control group. At certain locations, presence of intranuclear eosinophilic material could be nuclear edema or nuclear invagination or incidental finding. In CHA group, at 1 DPI, pancreas was intact, multifocal lymphoid nodules in the proximity of branches of pancreatic ducts, and occasionally, hyperplasia in the endocrine pancreas was seen. Rarely, cells in the acinar tissue had eosinophilic intranuclear material. This was only observed in the endocrine tissues. There was mild perivascular edema. Rare multifocal apoptotic cells (rounded, hypereosinophilic with pyknotic nuclei) were noticed. At 3 DPI, in CHA group, eosinophilic intranuclear material was noticed in endocrine pancreas. Multifocal to diffusely, there was partial depletion of zymogen granules form the exocrine pancreas. Acinar cells had occasional single to multiple vacuoles in the apical portion of the cell. Multifocally, there was mild perivascular edema and occasionally individualization of acinar cells. Few acinar cells undergo single cell necrosis/apoptosis and other cells had mild cytoplasmic vacuolation. No significant changes were observed in the endocrine pancreas. Occasionally, there was lymphocytic infiltration in the exocrine tissue. Rarely, there were eosinophilic intranuclear inclusion bodies in the endocrine tissue (Figure 2, E). At 5 DPI, in CHA group, exocrine cells had a complete loss of zymogen granules, rounded off, and separated from neighboring cells. The exocrine and endocrine tissues had lost the original structure, and cells were individualized. There was sever congestion in the large and small vessels. The epithelial cells of pancreatic duct were sloughed and lost nuclear detail as well. There was mild perivascular edema. Occasionally single cell necrosis (Figure 2, H). There was cytoplasmic vacuolation of acinar cells in the exocrine tissue and partial depletion of zymogen granules.

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In VAC+CHA group, at 1 DPI pancreatic tissue shad, multifocal, partial to complete depletion of zymogen granules, cytoplasmic vacuolation (degeneration) and sometimes necrosis of individual acinar cells of pancreas. At 3 DPI, the acinar cells undergo a spectrum of changes that range from degeneration, with loss of zymogen granules, cytoplasmic vacuolation and swelling of the cells, to single cell necrosis, with cells becoming individualized, and shrunken with pyknotic nuclei. There were multifocal, randomly distributed lymphoid nodules. Whereas at 5 DPI, partial or complete depletion of zymogen granules were observed. Frequently, cytoplasmic vacuolation with eosinophilic hyaline inclusion in acinar cells of pancreas were seen throughout the exocrine part. There were multifocal lymphoid nodules throughout exocrine pancreas. There was marked increase in the cytoplasmic vacuolation and single cell necrosis of acinar cells and due to partial depletion of zymogen granules, acinar cells looked ductless. There is mild to moderate interstitial edema in exocrine portion of pancreas and separation of acinar cells from neighboring cells. One focal area of lymphocytic infiltration in the peripancreatic duct branch, perivascular edema was observed. Persistently, scattered lymphocytes were observed throughout the exocrine pancreas. Most of the observed lesion were perivascular in this slide (Figure 2**Error! Reference source not found.**, I). Individualized round acinar cells with dark pyknotic nuclei showed sever single cell necrosis throughout the exocrine tissue. There was distortion of the acinar cells and separation from the neighboring cells. Diffusely, the areas of cytoplasmic vacuolation of acinar cells are seen.

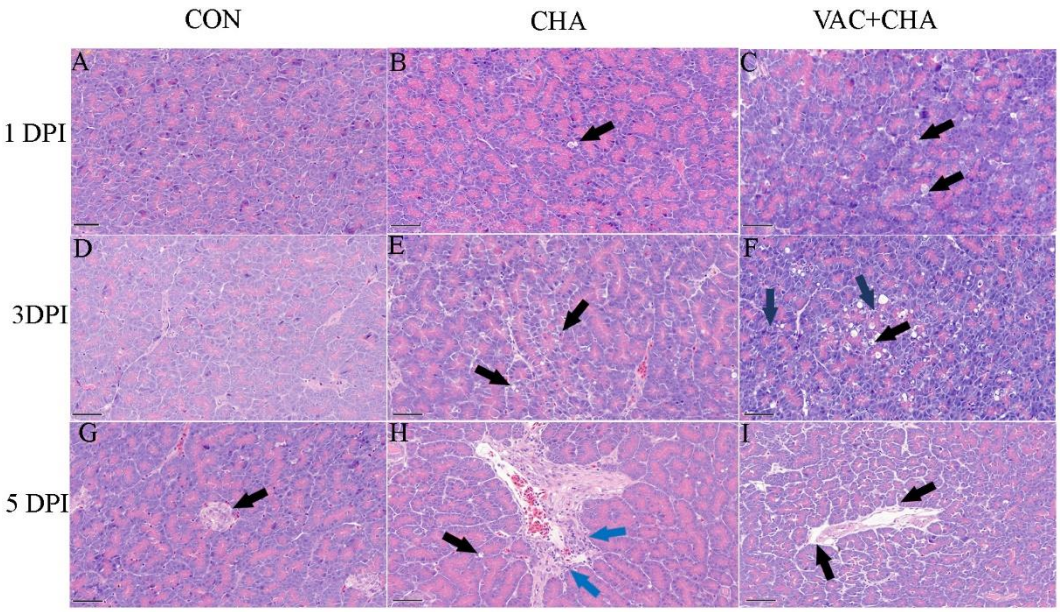

Figure 2. This Histopathological changes induced by the Newcastle disease virus in the pancreas of SPF and vaccinated chicken. Panels A-C, D-E, and G-I show the histopathology of control, NDV infected (CHA), and vaccine+NDV (VAC+CHA) infected groups at 1, 3 and 5 days post infection (DPI), respectively. Histopathological changes were not found from the control group. Sever histopathological changes were induced by the NDV infection in SPF chickens including B) Single cell; E) Cytoplasmic vacuolation and single cell necrosis (arrow); and H) Single cell necrosis (black arrow) and perivascular lymphocytic infiltration (blue arrows). Whereas, in vaccinated chickens histopathological changes induced by the NDV challenge includes C) Individualized shrunken cells F) Black arrow shows single cell necrosis and blue arrow shows individualized and shrunken cell and I) Mild perivascular edema at 5 DPI. Panels in the control group do not show any pathological change but unaffected endocrine pancreatic cells from the control group (G) are marked with arrow heads.

3.3. Apoptosis in the pancreas

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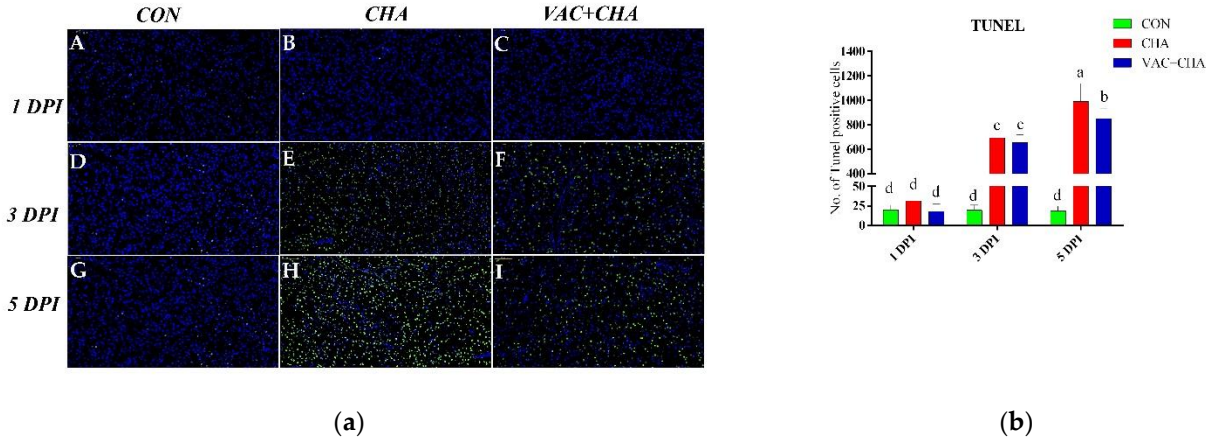
To estimate NDV induced apoptosis in the chicken pancreatic tissue, TUNEL positive cells were counted (Figure 3**Error! Reference source not found.**). The apoptotic cells had green stained nuclei. As presented in the Figure 3 A, the number of apoptotic positive cells in the pancreas was similar between the CON and NDV infected groups at 1 DPI. In the NDV challenged groups, the number of apoptotic cells were significantly increased compared to CON at 3DPI. The increase in the number of apoptotic cells was less evident in the VAC+NDV group compared to CHA, but, still it was significantly higher than the CON (Figure 3, B) at 5 DPI.



(**a**) (**b**)

Figure 3. T Fluorescent micrographs of chicken pancreas infected with NDV and stained by TUNEL assay technique. Panel A, D, and G represent the control (CON) group at 1-, 3- and 5- days post infection (DPI). Panel B, E and H represent the NDV challenged (CHA) SPF chickens. Panel C, F and I represent the vaccinated and NDV (VAC+CHA) challenge birds. Apoptotic cells show green nuclei whereas the nuclei in the normal cells shown in blue color.

3.4. Plasma concentrations of hormones

The plasma concentrations of CORT were significantly elevated ($P < 0.05$) in the CHA group compared to CON at 3 DPI. Significant difference was not found in the concentrations of CORT in CON and VAC+CHA (**Error! Reference source not found.**4, A). However, at 5 DPI, the plasma CORT level in CHA and VAC+CHA groups was significantly higher compared to the CON group.

Plasma concentrations of the glucagon and insulin are represented in the **Error! Reference source not found.**4 (B, C respectively). Statistically, non-significant effect of NDV challenge on glucagon was observed at 3 and 5 DPI, but numerically, slight decrease in the plasma levels of NDV infected birds were noticed compare to CON group. The plasma levels of insulin remained unaffected at 3 DPI, but significant decrease was observed in CHA group compared to control at 5 DPI (**Error! Reference source not found.**4, C).

Plasma concentration of the somatostatin is presented in the (Figure 4, D). The plasma concentrations of the somatostatin were significantly increased ($P < 0.05$) in CHA (from 261114c) group but, this increase was not significant in CON and VAC+CHA groups, at 3 DPI. Birds in the CHA group tends to have higher concentration ($P < 0.05$) of somatostatin at 5 DPI compared to CON and VAC+CHA groups.

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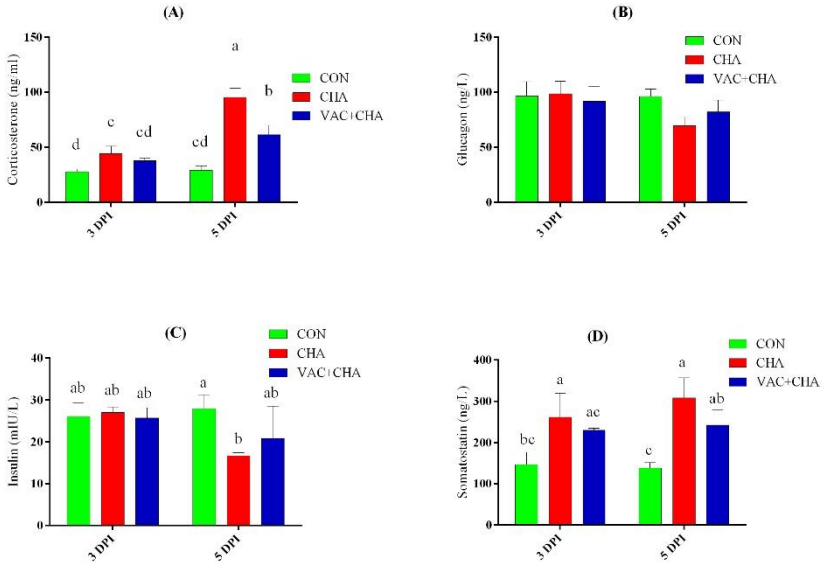

Figure 4. Effect of Newcastle disease virus infection on the plasma levels of Insulin (mIU/L), Glucagon (ng/L), Corticosterone (ng/ml), and somatostatin (ng/ml). The data are expressed as mean ± standard deviation. The bar with different letters are significantly different (ANOVA followed by Tukey's multiple comparisons test) at $P < 0.05$. CON, Control; CHA, Newcastle disease virus challenged; VAC+CHA, vaccinated with LaSota and challenged with virulent Newcastle diseases virus after 2 weeks of vaccination.is is a figure.

3.5. Activity and expression of pancreatic enzymes

The results for amylase activity and expression are presented in Figure 5 (A, B). At 3 DPI, chickens in the CHA group showed decreased activity of amylase ($p<0.05$), compared to the CON group. However, amylase activity in the pancreas of the VAC+CHA group of chickens was similar to that observed in the CON group, at 3DPI. More drastic decrease in the activity and expression of amylase in the pancreas of CHA groups of chickens were observed at 5 DPI compared to CON and VAC+CHA group. Furthermore, activity of amylase in the VAC+CHA was significantly less than control and, more than CHA group. The expression of amylase at 5 DPI from the CHA and VAC+CHA groups were significantly ($p<0.05$) less than the CON group.

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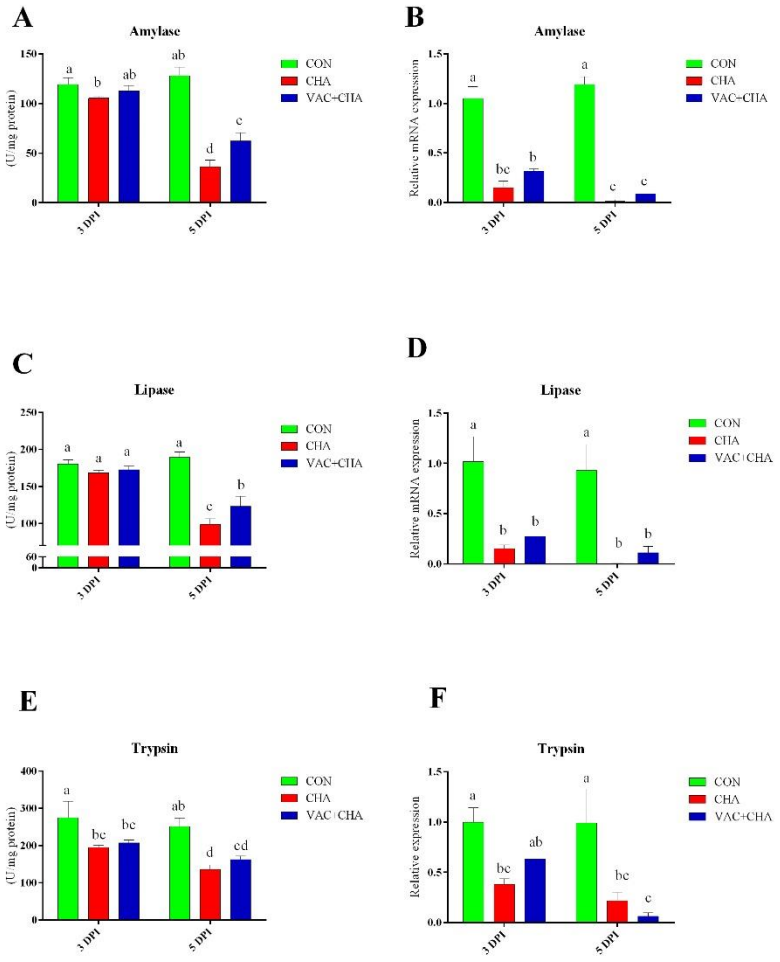

Figure 5. Effect of Newcastle disease virus challenge on the activity and expression of pancreatic enzymes. The data are expressed as mean ± standard deviation. The bar with different letters are significantly different (ANOVA followed by Tukey's multiple comparisons test) at $P < 0.05$. CON, Control; CHA, Newcastle disease virus challenged; VAC+CHA, vaccinated with Lasota and challenged with virulent Newcastle diseases virus after 2 weeks of vaccination.

There was non-significant difference in the activity of lipase at 3 DPI (Figure 5, C). NDV infection in SPF chickens resulted in decrease activity of lipase compared to chickens in the CON and VAC+CHA groups. Lipase mRNA expression in the pancreas had significantly decreased in NDV infected birds ($p<0.05$), compared to CON at 3, and 5 DPI (Figure 5, D).

The NDV challenge significantly affected the activity and expression of pancreatic trypsin (Figure 5, E, F). Activity of trypsin was significantly decreased in NDV challenge groups compared to CON, and this decrease was more obvious at 5 DPI. The mRNA expression of trypsin was significantly higher in the pancreas of CON birds ($p<0.05$), compared to CHA and VAC+CHA groups.

4. Discussion

The present study comprehensively reported the effect of NDV infection on the pancreas in vaccinated as well as SPF chickens. We persuasively demonstrated the replication of NDV in the pancreas of chicken in vaccinated and SPF chickens. In the study, chickens were vaccinated with 10^3 EID50 of the La Sota and challenged with $10^{6.5}$ EID50 of the ZJ1 after 2 week of vaccination because, at this dose of vaccine and challenge, pathogenic virus can replicate, can cause conjunctivitis and mild depression [27]. Histological studies of the chickens in the current experiment advocate the severe damage caused by

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the NDV in the pancreas. The NDV infection had caused the partial depletion to complete loss of zymogen granules form the exocrine pancreas, cytoplasmic vacuolation and necrosis of acinar cells cell, and eosinophilic intranuclear material was noticed in endocrine pancreas, but these were less evident at 3 DPI compared to 5 DPI. Histopathological results of the present study are in agreement with the previous studies [12,14,22-24].

The NDV infection in vaccinated as well as SPF chicken resulted in significant decrease in the activities and expression of pancreatic enzymes. These decreased activities and expression of amylase, lipase and trypsin may be related to extensive damage caused by the NDV to exocrine portion of pancreas in the infected birds. These decreased activities of the pancreatic enzymes may be one of the reason for poor feed conversion ratio in the NDV infected vaccinated birds. Decrease activities of the digestive enzyme may be related to poor production performance after NDV outbreaks in commercial poultry. Our hypothesis was that chickens having less vaccine dose can suffer with pancreatitis which may leads to decrease production of digestive enzyme.

It is well studied, that hypothalamic–pituitary–adrenal axis is involved in acute stress and regulate the inflammatory response to an infectious challenge. Stress is strongly correlated with the exacerbation of many viral infections [32,33]. In this study increased plasma levels of corticosterone were found in NDV infected chicken. Increased levels of corticosterone have been related to decrease growth rate, decreased antibody response, decreased number of lymphocytes, and reduced size of lymphoid organs [34]. Corticosterone decrease the insulin sensitivity in chicken [35]. In this study slight decrease in the plasma level of insulin and glucagon was noted at 5 DPI in NDV infected chicken. It may be due to less structural and morphological changes in the endocrine pancreas after NDV infection, as noted in the histopathology results. Avian pancreas has higher levels of glutathione, which exhibits protective effects on the beta cells [36]. This increased level of glutathione may be related to less pathogenicity in the endocrine pancreas because, decreased levels of glutathione was observed in the plasma and intestines of NDV infected chickens [6,37].

Previous studies had suggested the production of somatostatin (SS) from the endocrine cells in the proventriculus, small intestine [39], pancreas [40] and expression in the testis [41]. There are two forms of chicken somatostatin, somatostatin-14 and somatostatin-28, which are encoded by the same gene (PSS1) but another variant exist which is expressed in the brain and pancreatic islets [21,42]. The somatostatin inhibits the production of many hormones including insulin, glucagon, growth hormone, gastrin, vasoactive intestinal peptide, and thyroid-stimulating hormone [43]. In the present study, increase plasma levels of SS were found in NDV challenged birds. Increased somatostatin mRNA expression has been described after stimulation with cytokines in murine macrophage [44]. Similarly, increased plasma concentration of somatostatin in endotoxin injected sheep [45], septic pigs [46], simian immunodeficiency virus infected macaque, then infected with Mycobacterium Avium [47], increased expression in rabies virus infected brain [48] and secreted by human adipocytes after stimulation with activated macrophages, LPS, and IL-1β [49]. The increased plasma concentrations of somatostatin may be involved to counter the inflammation caused by infection. The SS has been described as anti-inflammatory and immunosuppressive and its analogues have been used as potential treatments for inflammation [50,51].

5. Conclusions

In summary, present study suggest that the pancreas could be a potential target of NDV infection. Evidently, exocrine pancreas is more severely affected by NDV compared to endocrine pancreas. The NDV infection leads to decrease in production and activity of digestive enzyme from the pancreas, even in the vaccinated chickens. Similarly, NDV infection also led to the increased levels of corticosterone and somatostatin, and decreased the production of insulin as evident from the plasma levels. These pathologies of

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the pancreas may be the leading cause of decreased production performance of NDV infected vaccinated birds.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Table S1. List of primer sequences used for quantitative PCR.

Gene Type	Forward Primer(5′-3′)	Reverse primer(5′-3′)	Amplicon size (bp)	Gen Bank accession number
ZJ1 M gene	ATGGACTCATCCAGGACAATCGGGCT	TTATTTCCTGAAAGGATTGTATTTAGCAATGG	1095	AF431744.3
ZJ1 M gene	TACTTTGATTCTGCCCTCCCTT	TAAGCAGAGCATTGCGGAAGA	255	AF431744.3
Trypsin	GTCGCCTTTGTTGGTGTGAC	GCTGATGAGAGAGCCTCCAC	150	NM_205385
Lipase	AGGAGAAGAAGGCTGGACCT	TCAGCACCTACAACACGGAC	141	XM_015288675
Amylase	TCAGGCTGGGAGGACATCTA	CCAGGGCCTGTTCGGATTAG	157	NM_001001473
GAPDH	CCATCACAGCCACACAGAAGAC	TGGACGCTGGGATGATGTT	93	NM_204305

Author Contributions:

Conceptualization, ZUR., C.M. and C.D.; methodology, X.Q., S.R., L.T., Y.L., C.S., W.L., Y.S., C.M.; formal analysis, Z.U.R., S.L.B., Z.M., SR, and J.I.; writing—original draft preparation, Z.U.R.; writing—review and editing, S.L.B., Z.M., J.I., C.M. and C.D.; supervision, C.M. and C.D.; funding acquisition, C.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All the animal experimental procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute (SHVRI, Shanghai, China), of the Chinese Academy of Agricultural Sciences (CAAS, Beijing, China). All protocols applied in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of SHVRI (Permission number: SHVI-RO-2018030189), CAAS, China. All efforts were made to minimize the suffering of experimental birds.

Informed Consent Statement: Not applicable

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

The appendix is an optional section that can contain details and data supplemental to the main text—for example, explanations of experimental details that would disrupt the flow of the main text but nonetheless remain crucial to understanding and reproducing the research shown; figures of replicates for experiments of which representative data is shown in the main text can be added here if brief, or as Supplementary data. Mathematical proofs of results not central to the paper can be added as an appendix.

Appendix B

All appendix sections must be cited in the main text. In the appendices, Figures, Tables, etc. should be labeled starting with "A"—e.g., Figure A1, Figure A2, etc.

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