Studies on the cellular responses of duck cells to Duck Tembusu virus and Japanese encephalitis virus infection and the effect of minocycline on Duck Tembusu virus-infected neurons

アヒルテンブスウイルスおよび日本脳炎ウイルス感染に対するアヒル細胞の応答ならびにアヒルテンブスウイルス感染神経細胞に対するミノサイクリンの効果に関する研究

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NOTES

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ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
Ara-C	Cytosine β-D-arabinofuranoside
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
BHK-21	Baby hamster kidney 21 cell
BSA	Bovine serum albumin
C	Capsid
CCK-8	Cell Counting kit-8
CNS	Central nervous system
Cx.	*Culex*
CPE	Cytopathic effect
DEDSV	Duck egg drop syndrome virus
DENV	Dengue virus
DMEM	Dulbecco's modified eagle's medium
DNs	Duck neurons
DTMUV	Duck Tembusu virus
E	Envelope glycoprotein
EDTA	Ethylenediaminetetraacetic acid

Env	Envelope protein
EtOH	Ethanol
FADD	Fas-associated death domain-containing protein/caspase-8 death signaling pathway
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	hour
HD	Hunting's disease
HIV	Human immunodeficiency virus
HPAI	Highly pathogenic avian influenza
h.p.i.	hour post-infection
IFNs	Interferons
IFN-α	Interferon-α
IFN-β	Interferons-β
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-1β	Interleukin-1β
ISGs	Interferon-stimulated genes
JAK	Janus kinase

JEV	Japanese encephalitis virus
MAV-1	Mouse adenovirus type 1
MEM	Minimum essential medium
MDA5	Melanoma differentiation factor 5
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MX	Myxovirus-resistance
NGF	Nerve growth factor
NS	Nonstructural protein
NSINV	Neuroadapted strains of Sindbis virus
OAS	Oligoadenylate synthetase
ORF	Open reading frame
PD	Parkinson’s disease
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming units
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PKR	Protein kinase R
prM	Precursor membrane

PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride membrane
qRT-PCR	Quantitative reverse transcription PCR
RABV	Rabies virus
RIG-I	Retinoic acid inducible gene-I
RNA	Ribonucleic acid
RNase-free	Ribonuclease free
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
SLEV	St. Louis encephalitis virus
STAT	Signal transducer and activator of transcription
SINV	Sindbis virus
TBEV	Tick-borne encephalitis virus
TBST	Tris-buffered saline with 0.05% Tween 20
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis inducing ligand
UK	United Kingdom

UTRs	Untranslated regions
USA	United States of America
WNV	West Nile virus
ZIKV	Zika virus

GENERAL INTRODUCTION

Duck Tembusu virus (DTMUV) is an important pathogen in a variety of avian species, including ducks, chickens, geese, sparrows, and pigeons [14, 33, 38, 40, 57, 85, 89, 92, 93]. This virus causes massive economic losses in poultry industries due to moderate to high morbidity and mortality, up to 90% and 5% to 30%, respectively [14, 85]. DTMUV was firstly diagnosed as a causative agent of ducks in duck-producing areas of China in April 2010 [14, 85]. This virus belongs to a mosquito-borne flavivirus [90]. Since the initial outbreak in China, it has emerged and reemerged in Southeast Asian countries, including Malaysia and Thailand [16, 39, 92]. Laying and breeding ducks show rapid egg production decline and reproductive problems [85, 106]. Infected ducks typically present moderate to severe neurological signs, including uncoordinated movement, wing and leg paralysis, circling, ataxia, and head tremor [16, 39, 85, 92]. Gross pathological findings of reproductive organs are hemorrhagic, distorted ovary, and regression of ovarian follicles. Histopathological examination mainly showed pathological changes in reproductive and neurological organs [85, 92]. Mononuclear cell infiltration in the interstitium and hemorrhage in ovarian follicles of DTMUV infected birds were recognized [14, 70, 85]. The CNS of affected birds represented non-suppurative encephalitis [14, 70, 85].

Japanese encephalitis virus (JEV) is a member of mosquito-borne flaviviruses and maintained in an environmental system through mosquito-bird or mosquito-pig transmission cycles [45]. This virus mainly causes encephalitis in humans and reproductive disorders in pigs [105]. Humans and horses are definitive hosts which showed fatal encephalitis [96]. Pigs have been considered as an important amplifying host of JEV for several decades, while many studies recently revealed that chickens, ducks, and pigeons can also act as amplifier of JEV especially in high density of poultry farming areas [4, 6, 59, 62]. Anti-JEV antibodies have been detected in a variety of free-ranging birds [68]. JEV-infected birds usually do not show

an clinical sign [46]. Infected adult birds normally presented only depression and decreased feed intake, and then they recovered within a short period [19, 45, 105]. JEV titers in the brain were detected and JEV antigen was also detected in neurons by immunostaining in the JEV-infected birds [26].

Despite DTMUV has been known as a neurotropic virus in birds. Studies on neuropathogenesis and immune responses against the virus in the central nervous system (CNS) are limited. JEV, another neurotropic flavivirus, is the most common cause of childhood viral encephalitis [44]. In contrast to DTMUV infection, birds infected with JEV typically show subclinical signs. Comparative studies on the mechanisms involved in viral-induced cell death and innate immune responses between DTMUV and JEV in duck cells will help us to understand the different pathogenicity of these two viruses in ducks. Based on these knowledges, it will provide valuable information for developing new treatment approaches. Until now, there has been no effective treatment for DTMUV infection. Neurological disturbance from DTMUV infection affects bird activities [92]. Poor feed consumption caused by neurological abnormality is contributed to retarded growth and possible susceptibility to secondary viral and bacterial infections [92]. Treatment against DTMUV-induced neurological symptoms can improve clinical outcomes in infected birds, which will be able to decrease morbidity and mortality rate. Hence, I conducted this research to elucidate the underlying mechanisms caused by DTMUV and JEV and to seek for effective treatments against DTMUV infection.

This thesis includes two chapters as follow:

Chapter I: Duck Tembusu virus induces stronger cellular responses than Japanese encephalitis virus in primary duck neurons and fibroblasts

Chapter II: Minocycline prevents primary duck neuron from duck Tembusu virus-induced death

CHAPTER I

Duck Tembusu virus induces stronger cellular responses than Japanese encephalitis virus in primary duck neurons and fibroblasts

INTRODUCTION

Duck Tembusu virus (DTMUV) is a contagious virus that severely reduces egg production [85, 92, 97]. Infected ducks show severe neurological signs, such as uncoordinated gait and a reluctance or inability to walk [85, 92]. The morbidity and mortality rates of infected ducks range up to 90% and 5% to 30%, respectively [14, 85]. This virus was first isolated from infected ducks in China in 2010 [85]. After the initial outbreak, it rapidly spread to other intensive duck farming countries including Malaysia [39] and Thailand [92]. Until now, no approved vaccinations or sufficient therapeutics have been developed. DTMUV is a positive-sense single-stranded RNA virus classified in the genus *Flavivirus* in the *Flaviviridae* family, which includes other human neuropathogenic flaviviruses such as Japanese encephalitis virus (JEV), West Nile virus (WNV), and tick-borne encephalitis virus (TBEV). Similar to other neuropathogenic flaviviruses, DTMUV can also cause encephalitis in a variety of animals, including mammals [97] and birds such as chickens [39], geese [40, 93], sparrows [89], and pigeons [57]. Once DTMUV enters the central nervous system (CNS) in infected birds, they typically show non-suppurative inflammation, including perivascular mononuclear cell infiltration and focal gliosis [70, 85, 92]. Immunohistochemical analyses demonstrated that DTMUV antigen can be detected in cytoplasm of Purkinje cells in the cerebellum of infected ducks [70]. Infected ducks also showed high expression of DTMUV RNA in the brains [70, 103]. This result indicates that duck neurons are the main targets of DTMUV infection and replication. Despite the importance of neuropathogenicity of DTMUV, the mechanism by which DTMUV causes neuronal cell death has not yet been investigated.

JEV is a zoonotic flavivirus that is transmitted by mosquitoes and vertebrates, including pigs and domestic birds [62]. Birds are principal amplifiers of JEV [5, 45, 73]. Clinical responses to JEV infection in birds are known to be age-related [19] although several studies

demonstrated that JEV infection in adult domestic ducks did not result in clinical outcomes [8, 19, 25, 26]. The clinical signs of infected ducklings are controversial. Clenton *et al*. reported that JEV-infected ducklings did not show apparent clinical signs [19]. In contrast, a recent study reported that some 2-day post-hatched ducklings infected with JEV showed mild neurological signs. Viral titers in their brains were detected and JEV antigens were detected in neurons by immunohistochemistry [26]. The pathogenesis of JEV in ducklings might be different depending on the JEV strains and route of infection [26, 48, 104]. Most previous studies on JEV have focused on infection in humans, pigs, and mosquitoes [2, 32, 37, 94]. It is known that JEV infects various mammalian neuronal cell types and causes neuronal death [47, 102, 107]. However, the pathogenesis of JEV infection in birds, including viral neurotropism and innate immune response has not been well investigated.

Although the disease outcomes of DTMUV- and JEV-infected birds are different, the biological consequences and underlying mechanism have not been fully understood.

Innate immune responses are the first line of host cell responses against viral infection [56]. Pattern recognition receptors (PRRs) consist of multiple family members, including toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I), and melanoma differentiation factor 5 (MDA5) [56]. The PRRs detect pathogens by recognizing structurally conserved molecules, subsequently inducing downstream signal transduction pathways, which activate expression of proinflammatory cytokines and type I interferons (IFNs), as well as the apoptosis pathway [56]. Although type I IFNs are known to be important components in counteracting virus infection, they play different roles in flavivirus infection. In WNV infection, type I IFNs not only limit viral replication but also protect neurons from cell death [81]. However, it has also been reported that WNV-infected neurons produce proinflammatory cytokines and chemokines, which promotes neuronal injury [53]. Neurons are the primary target of neurotropic viruses [53]. Neurons are known as a producer of proinflammatory cytokines and

chemokines, which promote neuronal injuries in infections with WNV [23], measles virus [101], rubella virus [101], human immunodeficiency virus (HIV) type 1 [36, 77] and Zika virus (ZIKV) [72]. Moreover, neuronal apoptosis has been recognized as one of the major hallmarks of neuronal pathogenesis in several neurodegenerative diseases, including viral infections [10, 74]. However, in DTMUV and JEV infection in ducks, it remains unclear whether neuronal apoptosis, or strong inflammatory response, or both are responsible for viral pathogenesis in the CNS.

Even though DTMUV is known to be a neurotropic flavivirus, the mechanisms by which DTMUV causes encephalitis in the CNS is unknown because of the lack of available duck neuronal cell lines. Previous studies on the pathogenesis and cellular responses in DTMUV infection have been performed using chicken or duck fibroblasts [17, 21, 86, 97, 109], but no information is available about the mechanisms of cell death and the innate immune responses during DTMUV infection in avian neurons. In addition, little is known about the response of avian neurons to JEV infection even though many bird species, including duck are susceptible to JEV. Therefore, studies using *in vitro* neuronal culture system are useful for investigating the neuropathogenesis of these viruses in ducks.

The first aim of this study was to understand the difference in the susceptibility of duck neurons to DTMUV and JEV. The second aim was to investigate neuro-specific innate immune response to DTMUV and JEV infection. Understanding the difference in the responses of duck neurons to DTMUV and JEV infection will provide useful information for understanding the difference in the pathogenicity of these viruses in infected ducks and may provide information for developing effective therapeutic strategies focusing on the mechanism of DTMUV-induced neuronal cell death in future.

MATERIALS AND METHODS

Cells and Viruses

The DTMUV strain, KPS54A61 (GenBank accession no. KF573582), was isolated from DTMUV infected ducks in Thailand in 2010 [16]. It was kindly provided by Prof. Thaweesak Songserm (Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand). The JEV strain, JaGAr-01, was isolated from *Culex* mosquitoes in Japan in 1959 [61] and stored in the laboratory of Comparative Pathology, Hokkaido University. Baby hamster kidney 21 (BHK-21) cells and a duck embryonic fibroblast cell line, CCL-141, purchased from the American Type Culture Collection (ATCC, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Biosera, Paris, France) and 2 mM L-glutamine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and its pH was adjusted with $NaHCO_3$. These cells were cultured at 37°C in 5% CO_2. Primary duck embryonic neurons (DNs) were prepared from 10- to 13-day-old duck embryos. Duck neuron culture protocol was modified from a primary chicken neuron culture method using dissected brains prepared by sterile procedure [52]. The brains were digested with 0.25% trypsin-EDTA solution (FUJIFILM Wako Pure Chemical Corporation) and the neurons were plated on tissue culture plates coated with poly L-lysine solution (Merck Millipore, Billerica, MA, USA). DN cells were grown in Neurobasal medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20 ng/µl native mouse nerve growth factor (NGF), 2.5S protein (Alomone Labs, Jerusalem, Israel) and 2% NS supplement (FUJIFILM Wako Pure Chemical Corporation). DN cells were maintained at 37°C in 5% CO_2 for 3 days, and then the old culture medium was replaced with fresh neurobasal media supplemented with 1 µM cytosine β-D-arabinofuranoside (Ara-C;

Sigma-Aldrich, St. Louis, MO, USA). After being cultured for 6-7 days *in vitro*, the resulting cells were used for experiments.

Plaque assay

Plaque assay was performed using BHK-21 cells in triplicate. Infection was performed for 1 h. Afterwards, the culture medium was removed and washed with PBS. Then overlay medium (1.25% methyl cellulose and MEM supplemented with 5% FBS) was added. Cells were incubated for 4 days and then fixed with 10% formalin and stained with 1% crystal violet in 70% ethanol.

RNA isolation and cDNA preparation

Cells were collected and total RNA was isolated with Tripure (Merck Millipore) and chloroform according to the manufacturer's protocols. RNA samples were treated with recombinant DNase I (RNase-free) (Takara, Shiga, Japan) to remove genomic DNA and subsequently transcribed to cDNA using PrimeScript 1st strand cDNA synthesis kit (Takara) according to the manufacturer's protocols. cDNA samples were synthesized from 25 ng of total RNA.

Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed in triplicate using KAPA SYBR FAST PCR Master Mix ABI Prism (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions. The signals were detected by Step One Real-Time PCR system (Thermo Fisher Scientific). qPCR conditions included 95°C for 3 min and 40 cycles of amplification at 95°C for 3 s and

60°C for 34 s. Lastly, melt curve analysis was carried out to confirm the specificity of the primers. The primers used in this experiment are shown in Table 1 [1, 56]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal amplification control.

Immunofluorescence Staining

For neuronal cell detection, DN cells at 6 or 7 days *in vitro* were fixed in 4% paraformaldehyde for 15-20 min at room temperature (RT) and then permeabilized with ice-cold 0.25% Triton X-100 in PBS for 5 min at RT. Cells were incubated with anti-β III tubulin antibody (5 µg/mL) (#18207; Abcam, Cambridge, UK) at 4°C overnight. On the next day, the cells were incubated with donkey anti-rabbit IgG Alexa-fluor-488 (1:5,000) (#21206; Thermo Fisher Scientific) and Hoechst 33342 (1:10,000) (Merck Millipore) at 37°C for 1 h. The cells were observed using an inverted fluorescence microscope (Keyence, Osaka, Japan).

Cell Viability Assay

CCL-141 and DN cells at 6-7 DIV were cultured in 96-well plates. On the next day, they were infected with DTMUV (KPS54A61) or JEV (JaGAr-01) at a multiplicity of infection (MOI) of 1. The cell viabilities of the infected cells were examined at serial time points [12, 24, 48, and 72 h post-infection (h.p.i.)] using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer's instructions.

Flow cytometry analysis

Apoptotic states at 6-7 days *in vitro* in CCL-141 and DN cells infected with DTMUV KPS54A61 or JEV JaGAr-01 (MOI = 1) were analyzed with BD FACS Verse system (BD

Bioscience, New Jersey, USA) using FITC Annexin V detection kit I (BD Biosciences) according to the manufacturer's instructions. Briefly, cells (1×10^6) were stained using the FITC Annexin V and propidium Iodide (PI) solution to detect apoptotic cells and measure cell death, respectively. In total 10,000 cells were analyzed per measurement. The data was analyzed using FCS expression 4 software (De Novo software, California, USA).

Immunoblotting analysis

Cell samples were harvested at different time points using RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% DOC, 0.1% SDS and 1% NP-40) containing a complete protease inhibitor cocktail (Merck Millipore). Proteins were separated by polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore). The membranes were incubated with anti-cleaved caspase-3 (5 μg/mL) (#2302, Abcam) or anti-β-actin antibodies (1:1,000) (#4970, CST). Anti-Mouse IgG antibody HRP-Linked Whole Ab Sheep (1:10,000) (GE Healthcare, Chicago, IL, USA) and anti-Rabbit IgG HRP-Linked Whole Ab Donkey (1:10,000) (GE Healthcare) were used as secondary antibodies. Chemiluminescent signals were developed with Immobilon Western HRP substrate (Merck Millipore) and detected using LAS mini 4000 (GE Healthcare UK Ltd., Little Chafon, England).

Caspase-3 inhibition

CCL-141 cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates for CCK-8 assay or 2×10^5 cells per well in 12-well culture plates for flow cytometry. On the next day, these cells were pretreated with 100 μM caspase-3 inhibitor Z-DEVE-FMK (Medical and Biological Laboratories, Nagoya, Japan) or culture media (untreated control) for 1 h. Then, cells were infected with DTMUV (KPS54A61) or JEV (JaGAr-01) at MOI=1 and

incubated at 37°C for 1 h. After viral infection, free viruses were removed by washing with PBS. The cells were incubated with the same concentrations of caspase-3 inhibitor or culture media. At 48 h.p.i., cell viability was measured by CCK-8 assay (Dojindo Molecular Technologies Inc.) and the numbers of apoptotic cells were measured by flow cytometry as described above.

Statistical analysis

Statistical analysis was performed using SPSS version 22 (SPSS Inc., Chicago, IL, USA). Normality of variables was checked by Shapiro-Wilk test. Viral titers of DTMUV and JEV, and the percentage of cell viability after DTMUV and JEV infections were compared by independent *t*-test. While comparisons of the apoptotic cell number and levels of mRNA expression were analyzed by mixed between-within-subjects analysis of variance. The analytical model included the effects of virus types, post-viral infection times, and the interaction between viruses and times. When the difference in types of viruses or the post-infection times or the interaction between viruses and times was significant, Bonferroni was used for further multiple comparison tests and simple effects analysis. The significant level was considered when $P < 0.05$.

RESULTS

DTMUV proliferate more than JEV in CCL-141 and DN cells

The primary culture plates containing 80-90% β-III tubulin positive cells were used for further experiments (Figs. 1A and 1B). To confirm that DNs and CCL-141 were susceptible to DTMUV or JEV infection and replication, they were infected with either virus at MOI=1. DTMUV infection in DNs and CCL-141 induced cytopathic effects (CPE) such as cell detachment and cell aggregation at 24 h.p.i. (Figs. 1C and 1D), and most of the infected cells were detached from culture plates at 72 h.p.i. (data not shown). JEV infection in DNs and CCL-141 also showed CPE, but morphological changes were milder than DTMUV infection at 24 and 48 h.p.i. Susceptibilities of DNs and CCL-141 to DTMUV and JEV were compared by analyzing the productivity of viral progeny. Cell culture supernatants were collected at several time points (12, 24, 48, and 72 h.p.i.) and plaque formation units were calculated to determine virus titers. The viral titers of DTMUV and JEV were increased in a time dependent manner up to 48 h.p.i., and DTMUV titer was higher than that of JEV at 12 and 72 h.p.i. in CCL-141 cells (Fig. 2A). Interestingly, DTMUV had high titers in DNs from the early time point, and JEV titers caught up with the same level as DTMUV at 24 h.p.i. (Fig. 2B). Furthermore, DTMUV indicated the highest titer at 48 h.p.i. These results suggest that both DNs and CCL-141 are permissive to DTMUV and JEV replication and that DTMUV can produce more viral progeny than JEV in both cell types especially at 12 h.p.i. (Figs. 2A and 2B).

DTMUV and JEV induce apoptosis in DN and CCL-141

To determine whether DTMUV or JEV growth result in cell death in DNs and CCL-141, cell viabilities were examined at 8, 12, 24, 48, and 72 h.p.i. DTMUV infection caused lower percentages of cell viabilities in both DNs and CCL-141 than JEV infection, which was obvious after 24 h.p.i. (Figs. 2C and 2D). Nuclear morphology of CCL-141-infected with DTMUV or JEV (MOI=1) was analyzed with Hoechst. At 48 h.p.i., virus infected cells showed chromatin condensation and nuclear fragmentation (Fig. 3A). Furthermore, DTMUV- or JEV-infected cells were subjected to Annexin V and PI staining followed by flow cytometry analysis. Both DTMUV and JEV induced apoptosis in CCL-141 and DNs at 24 h.p.i., but the percentages of apoptotic CCL-141 and DNs were significantly higher in DTMUV than in JEV (Figs. 3B-3E). The protein expression levels of cleaved caspase-3, a downstream executioner of apoptosis, were also analyzed. The results showed that DTMUV and JEV induced caspase-3 cleavage in CCL-141 and DNs at 48 h.p.i. and 72 h.p.i. The protein levels of cleaved caspase-3 in DTMUV-infected cells were higher than those in JEV-infected cells (Figs. 4A and 4B). Caspase-3 inhibitor treatment partially increased cell viability and decreased the number of apoptotic CCL-141 cells after infection with DTMUV and JEV (Figs. 5A-E).

These results suggest that DTMUV induces apoptosis in CCL-141 and DNs more strongly than JEV, and that the induced apoptosis in DTMUV or JEV-infected cells is mediated by caspase-3-dependent pathway.

DTMUV infection induced higher mRNA levels of innate immunity-related genes than JEV infection

The expression of PRRs (*RIG-I, MDA5*, and *TLR3*) in CCL-141 and DNs inoculated with DTMUV or JEV was initially examined. In CCL-141, mRNA expression levels of *RIG-I*, *MDA5*, and *TLR3* by DTMUV infection were significantly higher than those by JEV infection (Fig. 6A). In DNs, the expression of these genes did not show clear differences between DTMUV or JEV infection (Fig. 6B). There was a statistically significant difference in *RIG-I* expression at 48 h.p.i in DNs; however, this difference was still less than 2-fold. To compare the induction of innate immune responses in CCL-141 and DNs by inoculation with DTMUV or JEV infection, I analyzed mRNA expression levels of *IFN-α* and *IFN-β* genes. In both CCL-141 and DNs, expression levels of *IFN-α* and *IFN-β* by DTMUV infection were significantly higher than those by JEV infection (Figs. 6A and 6B). The expression levels of interferon-stimulated genes (ISGs) (*MX*, *OAS*, and *PKR*) were similar patterns of those of IFN mRNA expression. In CCL-141, DTMUV induced high levels of ISG expression at 24 h.p.i., while these genes were delayed expressed by JEV, however, they reached similar levels as DTMUV at 48 h.p.i. (Fig. 6A). In DNs, *OAS and PKR* expression was increased in DTMUV infection at 48 h.p.i., but JEV infection did not show an increase in these mRNAs in at 48 h.p.i. (Fig. 6B).

These results suggest that DTMUV induces more intense innate immune responses in both CCL-141 and DNs than JEV.

DISCUSSION

Similar to other flaviviruses causing encephalitis, the main target of DTMUV is neurons. However, the mechanism causing neuronal cell death in birds has not been determined. This study indicates that DNs was highly permissive for DTMUV and JEV infection and replication. DTMUV infection can strongly induce innate immune gene mRNA expression rather than JEV infection in CCL-141 and DNs. These findings suggest that DTMUV replication induced cell death and innate immune response more strongly than JEV in duck cells.

A previous study demonstrated that JEV induced apoptosis in SH-SY5Y human neuroblastoma cells *via* the activation of the caspase-3/7 pathway in a time-dependent manner [102]. Similarly, the present study found that DTMUV and JEV could induce apoptosis in DNs and CCL-141 probably *via* the caspase-3-dependent pathways (Figs. 3B-E, 4 and 5). Higher numbers of DNs and CCL-141 cells infected with DTMUV underwent apoptosis compared to the cells infected with JEV (Figs. 3D and 3E). These results were consistent with the protein expression levels of cleaved caspase-3 (Figs. 4A and 4B). Many reports have proposed that the viral proteins of flaviviruses exert a range of functions to counteract host cell antiviral mechanisms, including apoptotic mechanisms [3, 71]. The flavivirus proteins regulate apoptotic mechanisms either activation or inactivation in virus-infected cells [71]. WNV-capsid protein induces apoptosis *via* the importin-mediated pathway and the interaction of protein kinase C phosphorylation [11]. Controversially, the expression of WNV capsid protein enhanced phosphorylation of AKT, a serine/threonine-specific protein kinase, which can block the apoptosis pathway [95]. Airo *et al*. demonstrated that expression of capsid proteins derived from seven different flaviviruses also activate AKT survival pathway; although, each flavivirus capsid protein induced AKT phosphorylation at different levels [3]. Another study indicated that JEV-NS5 protein showed anti-apoptotic activity through the reduction of IFN-β-induced p38 MAPK/STAT1-mediated apoptosis [99]. As such, apoptosis regulatory by flaviviruses

proteins seems to be complicated [71]. Based on these previous observations, it is possible that some viral gene products of DTMUV and JEV may regulate the apoptotic pathway through either activation or inhibition. Further studies will be needed to fully elucidate the different pathogenesis between DTMUV and JEV in birds.

Although it has been documented that type I IFNs are essential for host cells to protect themselves from viral infections, the role of type I IFNs during infection varies between viruses. Clinical and animal studies on HIV-associated neurocognitive disorder have shown that an increased IFN-α production causes neuronal toxicity [49]. Meanwhile, IFN-α/β plays a role in protection of WNV-infected cells and results in preservation of neurons after inoculation of WNV [81]. In the present study, innate immune responses to DTMUV and JEV infection were compared in DNs and CCL-141 by qPCR analysis. DNs- and CCL-141 infected with DTMUV showed a significantly higher upregulation of PRRs, IFNs, and ISGs than those infected with JEV (Figs. 6A and 6B). This finding suggests that DTMUV induced stronger interferon signaling in duck cells than JEV. However, upregulated IFN signaling may not be efficient in controlling viral replication. Li *et al*. showed that DTMUV replicated quickly in several tissues, including spleen and brain. A high titer of DTMUV was obtained from the brain, where the mRNA of IFNs was highly upregulated [56]. Previous studies indicated that excessive inflammatory response and dysregulation of anti-inflammatory response (known as "cytokine storm") play significant roles in the pathogenicity of highly pathogenic avian influenza A (HPAI). Human and animal studies proved that HPAI induces high production of inflammatory cytokines and chemokines and this was consistent with severe clinical outcomes [9, 18, 24, 83]. A comparative study on host immune response to six HPAI strains in nonhuman primates suggested that H5N1 VN3040 strain (the most virulent strain among viruses in this study) attenuates induction of genes involved in innate immunity, apoptosis, and antigen processing/presentation in the early phase of infection. These resulted insufficient into

inhibition in viral replication [66]. In the late phase of infection, a subsequent continuous viral replication sustained induction of innate immune responses, including type I and III IFNs, and excessive inflammation. These further resulted into severe clinical signs in VN3040-infected animals. However, it is still unclear why the virus was able to replicate under the strong induction of innate immune responses [66]. The current study found out that CCL-141 infected with DTMUV induces mild levels of *IFN-α* and *IFN-β* mRNA expression at 24 h.p.i. and the levels of expression dramatically increased at 48 h.p.i. Therefore, it is possible that low levels of *IFN-α* and *IFN-β* mRNA expression at early phase of infection may not be efficient to suppress DTMUV replication. Moreover, a prolonged induction of innate immune responses at late stage of infection might cause tissue damage. In addition, several studies on neurological diseases and viral infections have indicated that increases in type I IFN production induce proinflammatory transcriptional signaling cascades in inflammation associated with neurological disease progression [79, 91]. A previous study also described how inflammation plays a critical role in the pathogenesis of several neurodegenerative diseases and disorders [50]. A clinical study indicated that the high mortality rates of JE patients were correlated with high cytokine concentrations in serum and cerebrospinal fluid [100]. Additionally, elevated levels of inflammatory cytokines and chemokines, IL-6, IL-8, and IFN-α can induce neuronal cell death following JEV infection [35, 100]. These previous findings collectively indicate the possibility that the higher IFN induction caused by DTMUV infection in duck cells may be involved in cell death due to the induction of strong inflammation.

In conclusion, the current study indicates that DNs are susceptible to both DTMUV KPS54A61 and JEV JaGAr-01 replication. DTMUV KPS54A61 infection induced apoptosis in higher numbers of DNs and CCL-141 than JEV JaGAr-01 infection *via* the caspase-3-dependent pathway. DTMUV KPS54A61 infection dramatically induced the rapid and robust mRNA expression of innate immune responses compared to JEV JaGAr-01 infection at early

infection. These results provide novel insights into the cell death mechanism and innate immune responses to DTMUV and JEV infection in ducks.

Fig. 1

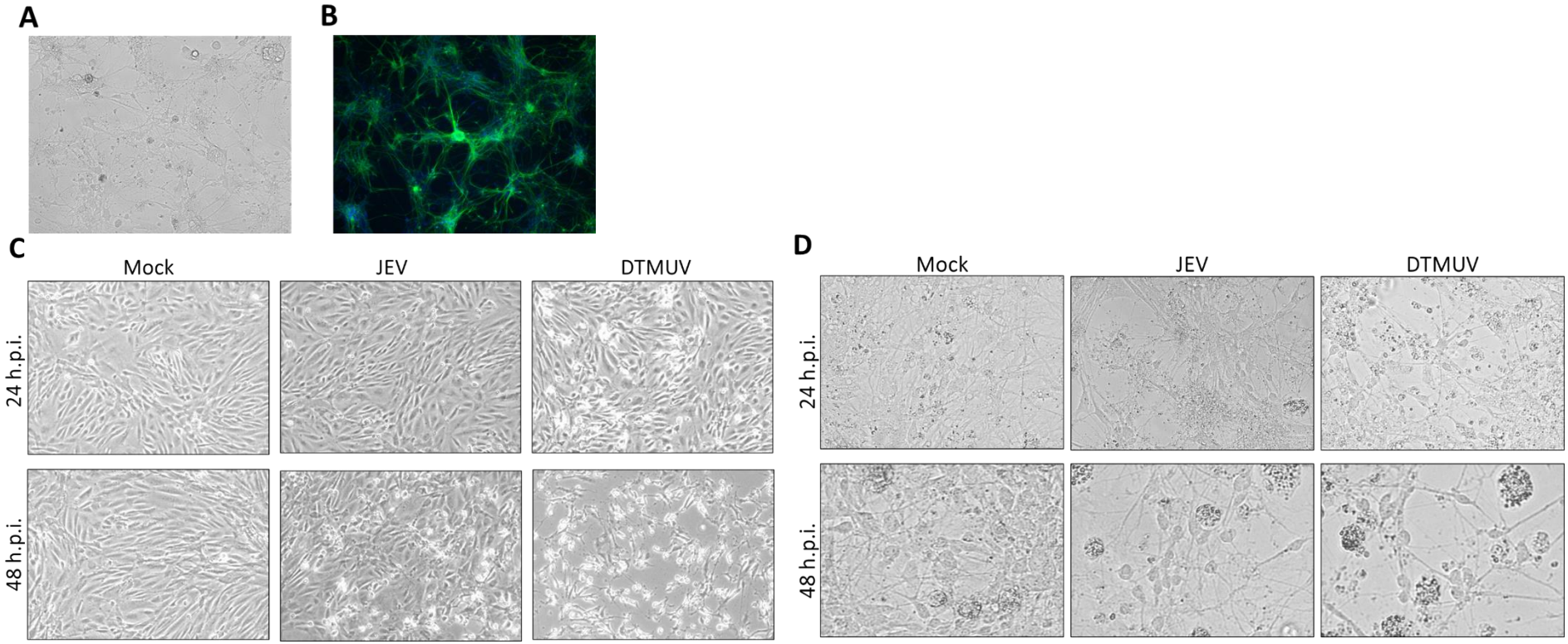

Fig. 1. DNs and CCL-141 morphologies in (A) primary culture of duck neurons (DNs) at day 5 day *in vitro* (DIV) (20×). (B) DNs demonstrated by indirect immunofluorescence using anti β-III tubulin antibody (green) at day 7 DIV. Cell nuclei were counterstained by Hoechst 33342 (blue). Morphological changes of CCL-141 (C) and DNs (D) at 24 h post-infection (h.p.i.). The cytopathic effects in CCL-141 and DNs occurred more rapidly induced by DTMUV than by JEV infection.

Fig. 2

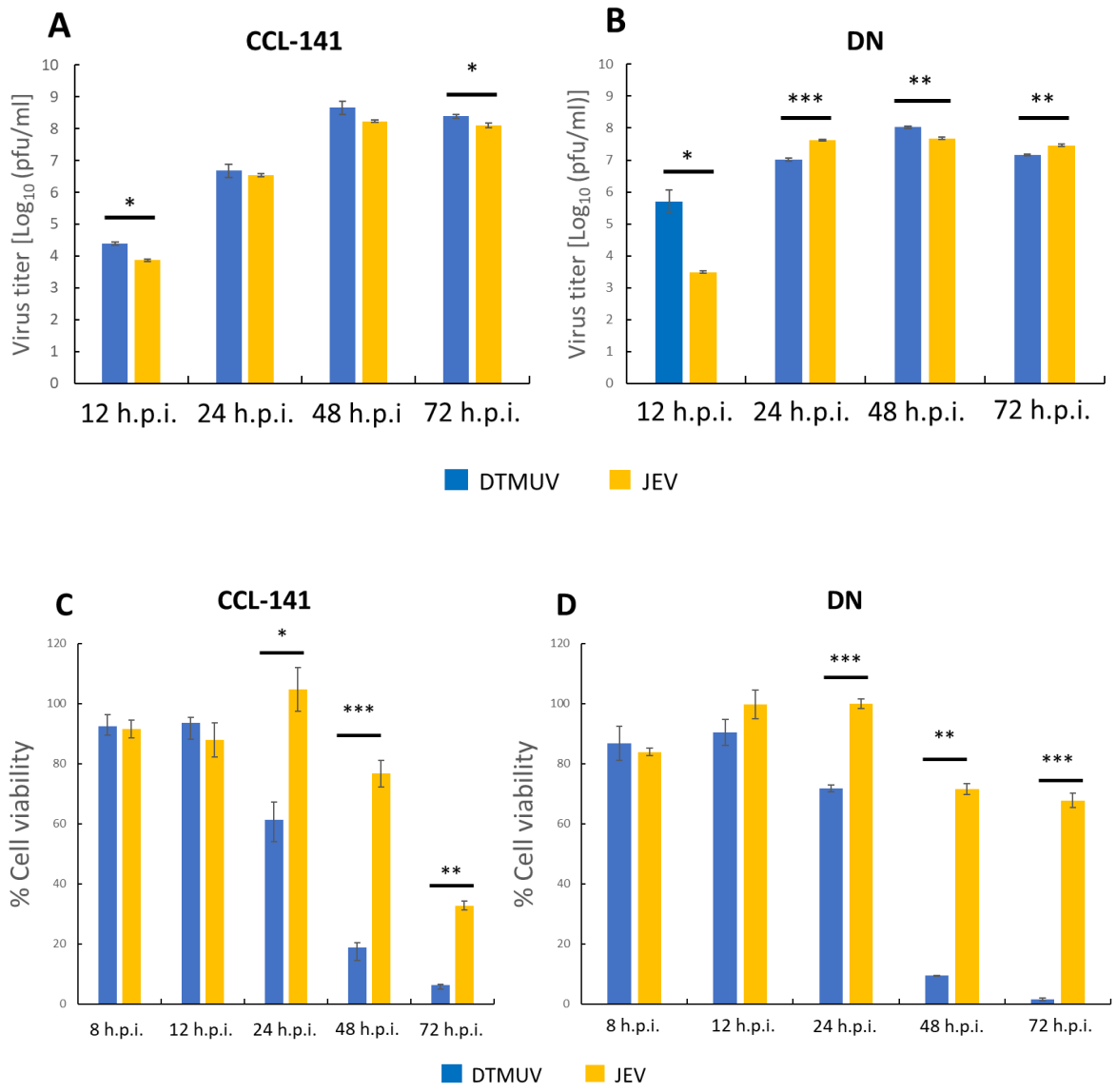

Fig. 2. Viral titers of DTMUV KPS54A61 and JEV JaGAr-01 in CCL-141 (A) and DNs (B). The percentage of cell viability of CCL-141 (C) and DNs (D)-infected with DTMUV and JEV. The results are shown as the mean ± SEM of three independent experiments. The asterisks indicate statistically significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; independent *t*-test)

Fig. 3

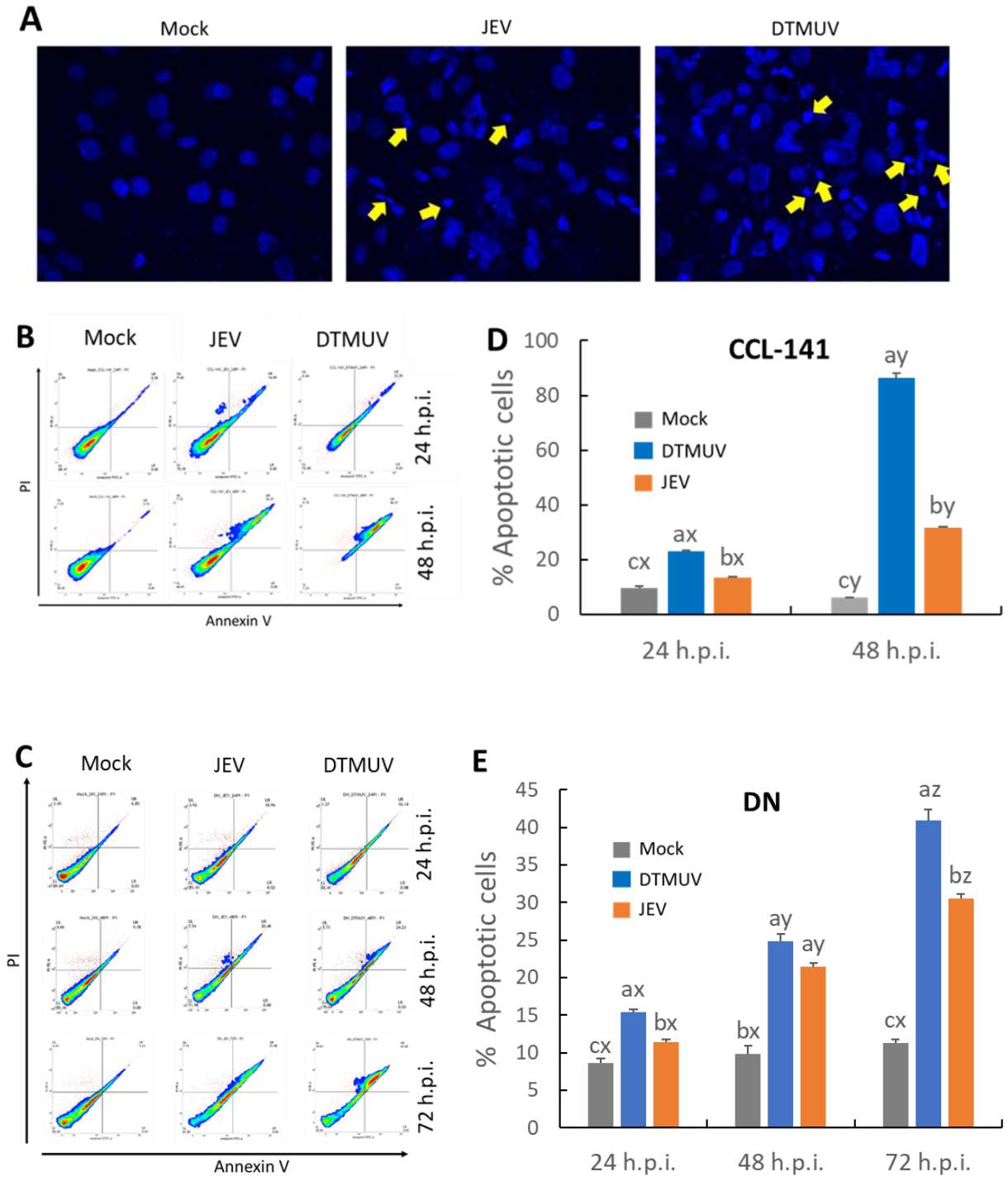

Fig. 3. DTMUV KPS54A61 and JEV JaGAr-01 triggered apoptosis in DNs and CCL-141. (A) Nuclei of CCL-141 were stained with Hoechst and observed under a fluorescence microscope. After 48 h.p.i.,

DTMUV and JEV caused DNs morphological changes including condensed-chromatin and nuclear fragmentation (arrows). (B) Representative data from three independent flow cytometry experiments demonstrating annexin-V and propidium iodide (PI) labels of Mock-, JEV- and DTMUV-infected CCL-141 cells at 24 and 48 h.p.i. (C) Representative data from three independent flow cytometry experiments demonstrating annexin-V and propidium iodine (PI) labels of Mock-, JEV- and DTMUV-infected DN cells at 24, 48 and 72 h.p.i. (D) The percentage of apoptotic cells of CCL-141 cells during DTMUV- and JEV infection. (E) The percentage of apoptotic cells of DN cells during DTMUV- and JEV infection compared with mock infection. The results are shown as the mean ± SEM of three independent experiments. x, y, and z indicate significant difference between time post viral infection ($P < 0.05$). a, b, and c indicate significant difference between DTMUV and JEV ($P < 0.05$).

Fig. 4

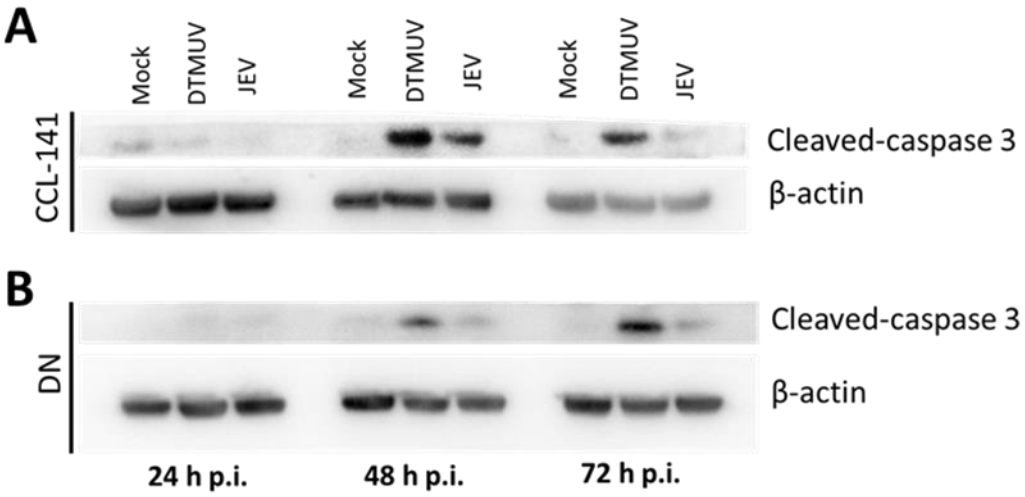

Fig. 4. Immunoblot analysis for cleaved caspase 3 in CCL-141 (A) and DNs (B) infected with DTMUV KPS54A61 or JEV JaGAr-01 at MOI=1. β-actin was used as the loading control. Total proteins were extracted at the indicated time points from treated-CCL-141 and DN cells.

Fig. 5

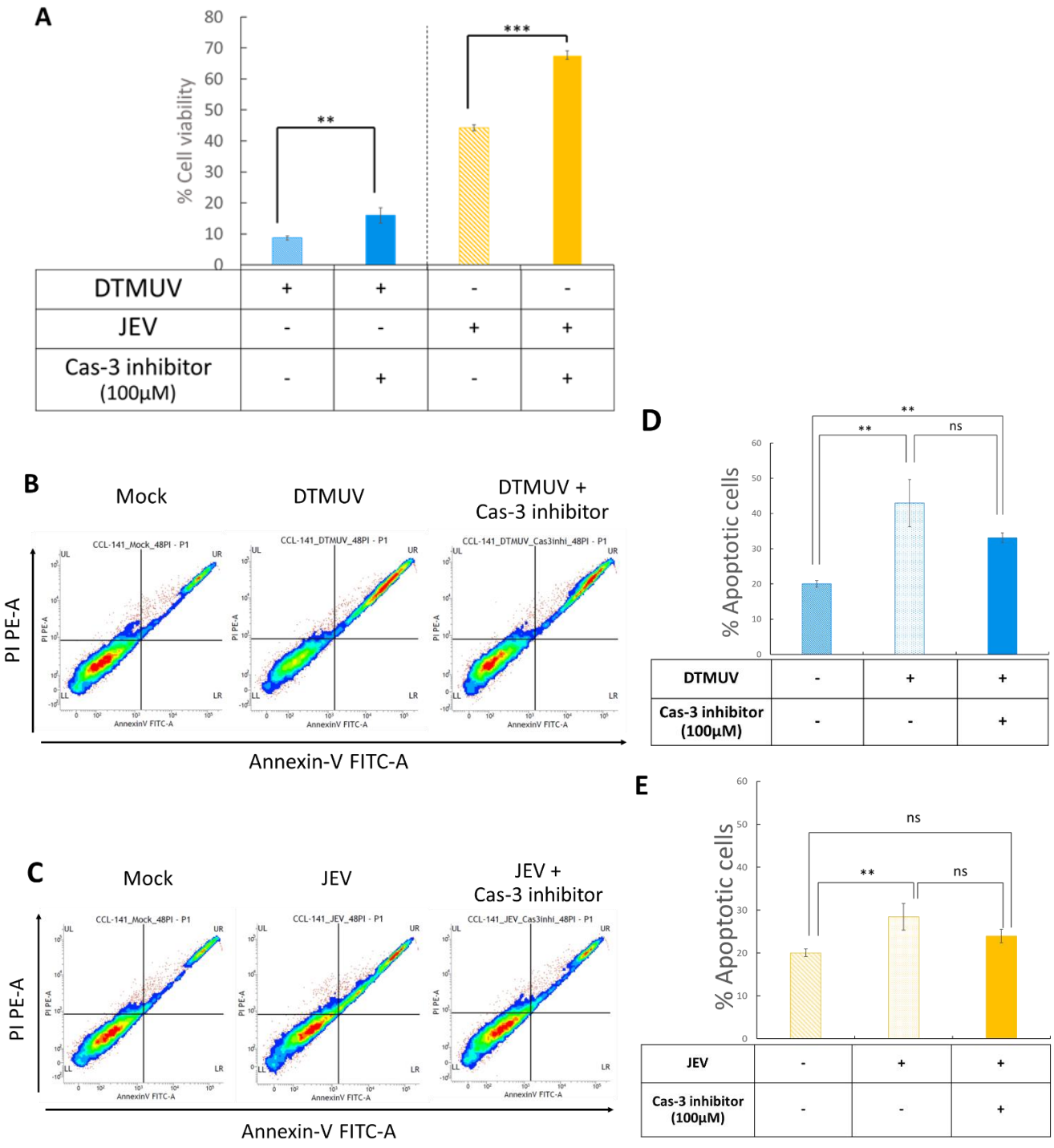

Fig. 5. Effects of a caspase-3 inhibitor on CCL-141-infected with DTMUV KPS54A61 or JEV JaGAr-01. (A) The percentage of cell viability of DTMUV- and JEV-infected CCL-141 with or without treatment of caspase-3 inhibitor (100 μM) at 48 h.p.i. (B and C) Representative data from three independent flow cytometry experiments demonstrating annexin-V and propidium iodide (PI) labels of

uninfected CCL-141 cells without treatment (Mock), virus-infected CCL-141 cells without treatment (DTMUV in B, JEV in C) and virus-infected CCl-141 cells with treatment of caspase-3 inhibitor (100 μM) (DTMUV + Cas-3 inhibitor in B, JEV + Cas-3 inhibitor in C) at 48 h.p.i. (D and E) The percentage of apoptotic CCL-141 cells during DTMUV (D) and JEV (E) infection in the presence or absence of caspase-3 inhibitor compared with mock infection at 48 h.p.i. The results are shown as the mean ± SEM of six independent experiments for cell viability analysis and three independent experiments for flow cytometry. The asterisks indicate statistically significant differences (** $P < 0.01$; *** $P < 0.001$; ns, not significant; one-way analysis of variance followed by Bonferroni multiple-comparison test).

Fig. 6

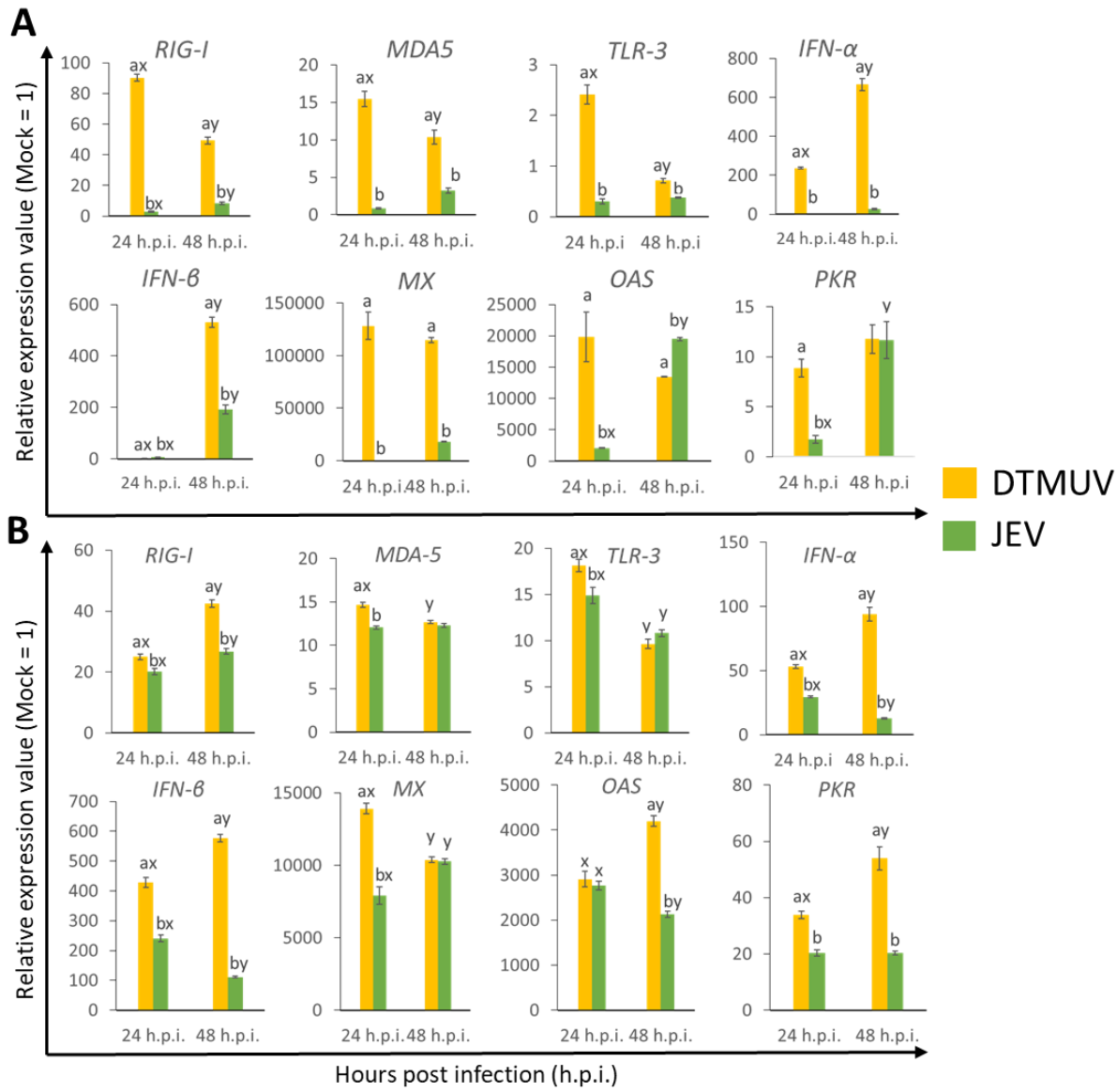

Fig. 6. Expression of immune-related genes in CCL-141 (A) and DNs (B) infected with DTMUV KPS54A61 and JEV JaGAr-01. The results were normalized using the *GAPDH* expression level. The expression levels among samples were compared using relative expression values by setting the expression levels in mock-infected samples at 24 h.p.i. as 1. The results are shown as the mean ± SEM of three independent groups. x and y indicate significant differences between times post-viral infection within the same virus infection ($P < 0.05$). a and b indicate significant differences between DTMUV and JEV within the same time post-viral infection ($P < 0.05$).

Table. 1

Primers used in this study

Primer name	Sequence (5'-3')	Product size (bp)	GenBank no.
RIG-I F	GCTACCGCCGCTACATCGAG	224	EU363349
RIG-I R	TGCCAGTCCTGTGTAACCTG		
MDA5 F	GCTACAGAAGATAGAAGTGTCA	120	KJ451070.1
MDA5 R	CAGGATCAGATCTGGTTCAG		
TLR3 F	GAGTTTCACACAGGATGTTTAC	200	JQ910167
TLR3 R	GTGAGATTTGTTCCTTGCAG		
IFN-α F	TCCTCCAACACCTCTTCGAC	232	EF053034
IFN-α R	GGGCTGTAGGTGTGGTTCTG		
IFN-β F	AGATGGCTCCCAGCTCTACA	210	KM035791.1
IFN-β R	AGTGGTTGAGCTGGTTGAGG		
MX F	TGCTGTCCTTCATGACTTCG	153	GU202170.1
MX R	GCTTTGCTGAGCCGATTAAC		
OAS F	TCTTCCTCAGCTGCTTCTCC	187	KJ126991.1
OAS R	ACTTCGATGGACTCGCTGTT		
PKR F	AATTCCTTGCCTTTTCATTCAA	109	Unpublished
PKR R	TTTGTTTTGTGCCATATCTTGG		
GAPDH F	ATGTTCGTGATGGGTGTGAA	176	AY436595
GAPDH R	CTGTCTTCGTGTGTGGCTGT		

SUMMARY

Duck Tembusu virus (DTMUV) and Japanese encephalitis virus (JEV) are mosquito-borne flaviviruses. These two viruses infect ducks; however, they show different neurological outcomes. The mechanism of DTMUV- and JEV-induced neuronal death has not been well investigated. The present study investigated the differences in the mechanisms involved in virus-induced cell death and innate immune responses between DTMUV KPS54A61 strain and JEV JaGAr-01 strain using primary duck neurons (DNs) and duck fibroblasts (CCL-141). DNs and CCL-141 were permissive for the infection and replication of these two viruses, which upregulated the expression of innate immunity genes. Both DTMUV and JEV induced cell death *via* a caspase-3-dependent manner; however, DTMUV triggered more cell death than JEV in both CCL-141 and DNs. These findings suggest that DTMUV infection causes apoptosis in duck neurons and fibroblasts more strongly than JEV. Levels of the mRNA expression of innate immunity-related genes by DTMUV infection were generally higher than levels by JEV infection, suggesting that DTMUV-induced immune response in duck cells may exhibit toxic effect rather than protective effects.

Chapter II

Minocycline prevents primary duck neuron from Duck Tembusu virus-induced death

INTRODUCTION

DTMUV is a mosquito-borne flavivirus that is mainly transmitted by *Culex tritaeniorhynchus* mosquitoes [82, 90]. It is a causative agent of severe neurological diseases in various bird species, including ducks, geese, chickens, pigeons, and sparrows [40, 57, 88, 89, 106]. Laying and breeding ducks show a sudden decline in egg production following infection with DTMUV [85]. Clinical signs of reproductive problems have been documented as hyperemic and flaccid ovaries and oophoritis [60, 70, 85]. Affected birds showed severe neurological symptoms such as unbalanced gait, wing and leg paralysis, and head tremor [70]. Histopathological analysis confirmed encephalitis composed of moderate to severe lymphocytic inflammation surrounding the perivascular spaces and diffuse glial nodules [60]. Furthermore, it was reported that high titer DTMUV was isolated from the CNS of infected birds [60, 70]. Hence, DTMUV is known as a neurotropic flavivirus and continuous to emerge and reemerge in several countries, including China, Malaysia, and Thailand [39, 69, 92]. However, no approved vaccines or therapeutic treatments are yet available.

Apoptosis normally occurs in tissue development, homeostasis in adult tissues, and defense mechanisms against pathogens [29]. However, inappropriate apoptosis is commonly associated with diseases such as cancer and neurodegenerative disorder [29]. Several neurotropic viruses also induce neuronal death *via* apoptosis, which plays a pivotal role in neuropathogenesis, concurrent with uncontrolled inflammatory responses [15]. I found that DTMUV can induce apoptosis in primary duck neurons (DNs), which is correlated with a reduction of DN cell viability after DTMUV infection (as described in Chapter I).

Minocycline is a second-generation semi-synthetic tetracycline derivative that is well-known as a bacteriostatic antibiotic which can crosses the blood-brain barrier [75]. It also exhibits properties such as anti-inflammatory, antioxidative, anti-apoptosis, and antiviral functions. Neuroprotective effects of minocycline have been documented in multiple

experimental studies and clinical researches, including Hunting's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and traumatic injury [34, 76, 78, 108].

Minocycline inhibits replication of several neurotropic viruses such as the JEV, WNV, dengue virus (DENV), Simian immunodeficiency virus (SIV), and human immunodeficiency virus (HIV) [13, 63, 65, 110]. An experimental study on minocycline treatment for JEV infected mice demonstrated significant decrease in viral replication, proinflammatory mediators, and neuronal apoptosis [64, 65]. In addition, Michaelis *et al*. showed that minocycline treatment inhibited the cytotoxicity of WNV to human neuronal cells, reduced the viral titer in a dose-dependent manner, and suppressed caspase-3 and poly (ADP-ribose) polymerase activation [63]. Furthermore, WNV-infected spinal cord slice cultures treated with minocycline showed decreased viral replication, proinflammatory microglial activation, and apoptosis in neurons [78]. Minocycline also exerted antiviral effects in mouse neuroblastoma Neuro2a infected with four different serotypes of DENV (DENV 1–4), although it failed to reduce the number of dead cells [55].

Neurological dysfunctions prevent DTMUV-infected birds from normal activity and get them emaciated. This makes them more susceptible to other viral and bacterial infections, resulting in high morbidity and mortality [97]. Minocycline has been preliminarily used in human clinical trials to treat neurological diseases based on the successful results in several experimental studies [42, 67]. Several studies have confirmed successful outcomes with minocycline in spinal cord injury, multiple sclerosis, and JEV infection [54, 75]. Considering the usefulness of minocycline for neurological diseases, I hypothesized that minocycline treatment can also treat neurological symptoms caused by DTMUV. Moreover, minocycline was possible to reduce reproductive problems caused by DTMUV. Based on the previous histological studies it is suggested that mononuclear cell infiltration in ovarian interstitium may play a role in the destruction of ovarian follicles of DTMUV-infected birds [14, 70, 85].

Therefore, an anti-inflammatory function of minocycline might have the potential to decrease pathological changes in reproductive organs of DTMUV-infected birds by repressing inflammatory responses.

Therefore, this study aimed to investigate the effects of minocycline on DTMUV infection. The effects of minocycline on DTMUV-infected DNs and the underlying mechanism of action were examined. This is the first study to investigate the efficacy of minocycline against DTMUV infection in duck neurons.

MATERIALS AND METHODS

Cell culture and Virus

Baby hamster kidney-21 (BHK-21) cells were purchased from the American Type Culture Collection (ATCC CCL-141, ATCC, Manassas, VA, USA) and cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Biosera, Nuaillé, France).

DNs were prepared from duck embryos at embryonic days 10–13. This culture protocol was modified from the long-term primary chicken neuron culture method, as previously described [52]. Briefly, brain samples were obtained from duck embryos and placed in Hanks' balanced salt solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA). Tissues were digested with a 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and gently triturated by pipetting in a neurobasal medium (Thermo Fisher Scientific) supplemented with 20 ng/μl native mouse nerve growth factor (NGF), 2.5S protein (Alomone Labs, Jerusalem, Israel), and 2% NS supplement (FUJIFILM Wako). Dissociated cells were seeded onto 12-well plates coated with a poly L-lysine (Merck Millipore, Billerica, MA, USA). Duck neurons were cultured at 37°C, and the old medium was replaced with fresh one supplemented with 1 μM cytosine β-D-arabinofuranoside (Ara-C; Sigma-Aldrich, St. Louis, MO, USA). After 6 to 7 days *in vitro*, DNs were used for further experiments.

The DTMUV strain KPS54A61 (GenBank accession No. KF573582) was isolated from the brain and spinal cord of infected ducks in Thailand and kindly provided by Prof. Thaweesak Songserm at the Faculty of Veterinary Medicine, Kasetsart University (Nakhon Pathom, Thailand) [16].

Immunofluorescence assay (IFA)

For neuronal cell detection, DNs at 6 or 7 days *in vitro* were fixed in 4% paraformaldehyde for 15–20 min at room temperature (RT) and then washed with 0.1 M glycine in phosphate-buffered saline (PBS) for 5 min. Cells were permeabilized with ice-cold 0.25% Triton X-100 in PBS for 5 min at RT, and then washed with a wash solution containing 0.05% Triton X-100 in PBS. The cells were blocked with 2% bovine serum albumin (BSA) at 37°C for 1 h. The blocking solution was removed and cells were incubated with anti-β III tubulin antibody (ab18207; Abcam, Cambridge, UK, 5 μg/ml) diluted in the blocking solution for overnight at 4°C. Following incubation, the cells were washed with wash solution and incubated with donkey anti-rabbit IgG Alexa Fluor 488 (#21206; Thermo Fisher Scientific, 1:5,000) and Hoechst 33342 (MERCK Millipore, 1:10,000) at 37°C for 1 h. The cells were washed twice with wash solution, and the signals were visualized using an inverted fluorescence phase-contrast microscope (BZ-9000; Keyence, Osaka, Japan) or confocal microscope (LSM-700, Carl Zeiss AG, Jena, Germany).

Plaque assay

The DTMUV titer was determined by a plaque assay using BHK-21 cells. Supernatants were collected from infected plates at 48 h post-infection (h.p.i.). In brief, 70 to 80% confluent BHK-21 cells were infected with DTMUV for 1 h. The plate was washed with PBS and overlaid with MEM supplemented with 5% FBS and 1.25% methylcellulose. After 72 h of incubation, the cells were washed with PBS, fixed with 10% formalin, and stained with 1% crystal violet in 70% ethanol. The number of plaques was counted under an inverted Eclipse TS 100 microscope (Nikon, Tokyo, Japan) and expressed as plaque-forming units (pfu/ml) were calculated. Independent triplicate wells were analyzed for statistical analysis.

Minocycline treatment

Minocycline (Sigma-Aldrich) was dissolved in sterile water and serially diluted in maintenance neurobasal media (vehicle). DNs at 6 to 7 days *in vitro* were pretreated with minocycline at different concentrations (0, 5, 10, 20, and 40 μg/ml) for 1 h, and infected with DTMUV at a multiplicity of infection (MOI) of 1 for 1 h. Finally, the medium was replaced with fresh medium containing the same doses of minocycline as pretreatment. Control cells were pretreated and treated with the vehicle. The minocycline treatment scheme is shown in Fig. 8A. The plates were incubated at 37°C in a CO_2 incubator until the end of experiments.

Cell viability assay

Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to analyze the percentage of viable DNs according to the manufacturer's instructions. DNs were cultured in 96-well plates at a density of 5×10^4 cells/well, treated with minocycline, and infected with DTMUV as described above. The percentage viability of DNs at several time points (12, 24, 48, and 72 h.p.i.) was calculated. Next, 10 μl of CCK-8 reagent was added to each well, and the cells were incubated in the dark at 37°C for 2 h. The absorbance was measured at 450 nm wavelength with a Nanodrop 2000 (Thermo Fisher Scientific).

Flow cytometry analysis

Apoptosis of DTMUV-infected DNs with or without minocycline treatment was analyzed using the fluorescein isothiocyanate (FITC) Annexin V detection kit I (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, cells were harvested at 24, 48, and 72 h.p.i. and stained with Annexin V and propidium iodide (PI) to detect apoptotic cells and measure cell death, respectively. Finally, the signals were detected

using a BD FACS Verse system (BD Biosciences). FCS expression 4 software (De Novo software, Pasadena, CA, U.S.A.) was used to analyze the data.

RNA isolation and cDNA preparation

At 48 and 72 h.p.i., total RNA was extracted from DTMUV-infected DNs with or without minocycline treatment using Tripure (Merck Millipore) and chloroform according to the manufacturer's protocols. Total RNA was treated with recombinant DNase I (RNase-free) (Takara Bio, Shiga, Japan), and cDNA was synthesized using PrimeScript 1st strand cDNA synthesis kit (Takara Bio) according to the manufacturer's protocols.

Quantitative reverse transcription PCR (qRT-PCR)

The qRT-PCR reaction was performed in triplicates on a Step One Real-Time PCR system (Thermo Fisher Scientific) using KAPA SYBR FAST PCR Master mix ABI Prism (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions. qRT-PCR data were analyzed using Applied Biosystems Step One Software (version 3.0; Thermo Fisher Scientific). The relative expression level was calculated using the delta-delta Ct method. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous amplification control for data normalization. Finally, melting curve analysis was carried out to confirm the specificity of the primers. The primers used for qRT-PCR are listed in Table 2 [1, 20, 56].

Statistical analysis

The significance of the differences among groups was evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple-comparison test. All statistical analyses were performed using SPSS (version 22.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Minocycline increased DN viability following DTMUV infection but did not affect DTMUV replication

The presence of DNs in culture plates was confirmed by an indirect IFA. As more than 70-80% cells in the plates expressed the neuronal marker β-III tubulin, these cells were used for further experiments (Fig. 7A). To investigate the cytotoxic effects of minocycline, DNs were treated with various concentrations of minocycline (0, 5, 10, 20, and 40 μg/ml) and their viability was analyzed at several time points (12, 24, 48, and 72 h.p.i.). The results showed that minocycline treatment had no effect on DN viability over the course of 72 h (Fig. 7B).

Next, to examine if minocycline can protect DNs from death induced by DTMUV infection, DNs were infected with DTMUV (MOI = 1) followed by treatment with different concentrations of minocycline. The cells were pretreated with minocycline for 1 h, infected with DTMUV for 1 h without minocycline, and then cultured for 12, 24, 48, and 72 h.p.i. with minocycline (Fig. 8A). DTMUV-infected DNs treated with 40 μg/ml minocycline showed similar morphology to DTMUV-infected untreated DNs. (Fig. 8B); however, minocycline treatment increased cell viability in a dose-dependent manner (Fig. 8C). Although there was no effect of minocycline on DN viability at 12 and 24 h.p.i. at all concentrations, 20 and 40 μg/ml minocycline concentrations significantly increased cell viability at 48 and 72 h.p.i. and 10 μg/ml minocycline treatment showed a significant increase in cell viability at 72 h.p.i. (Fig. 2C).

To investigate the effect of minocycline on DTMUV replication, DTMUV titers were compared between minocycline treatment groups (20 and 40 μg/ml) and non-treatment group at 48 and 72 h.p.i. Minocycline treatment did not inhibit DTMUV replication in infected DNs. Surprisingly, the viral titers in DTMUV-infected DNs treated with minocycline were

significantly higher than those in DTMUV-infected DNs without minocycline treatment at 48 and 72 h.p.i. (Fig. 8D).

These results suggest that minocycline decreased the number of DTMUV-infected dead DNs in a dose-dependent manner but had no antiviral effect on DTMUV replication.

Minocycline inhibits DTMUV-induced DN apoptosis

To examine the anti-apoptotic effects of minocycline in DNs, the number of apoptotic cells was analyzed by flow cytometry using Annexin V and PI staining. At 24 h.p.i., there was no statistical difference in the number of apoptotic cells between DTMUV-infected DNs treated with 0, 20, and 40 μg/ml minocycline and noninfected DNs (Fig. 9) At 48 h.p.i., the proportion of apoptotic cells was comparable between minocycline-treated and non-treated DTMUV-infected DNs. However, at 72 h.p.i., the minocycline-treated groups showed decreased number of apoptotic cells. Furthermore, 40 μg/ml minocycline treatment significantly reduced the number of apoptotic cells than 20 μg/ml minocycline treatment. These results indicate that minocycline as an inhibitor for apoptotic cell death and it works in a dose-dependent manner, consistent with the survival rate of cells observed in the cell viability assay (Fig. 8C). Therefore, minocycline exerts a neuroprotective role in neuronal apoptosis.

Minocycline alters the expression of inflammatory genes in DTMUV-infected DNs

To determine the anti-inflammatory effects of minocycline in response to DTMUV infection, the expression levels of inflammatory genes in DTMUV-infected DNs were examined in untreated or treated with 20 and 40 μg/ml minocycline at 48 and 72 h.p.i. using qRT-PCR. At the mRNA level, the expression of *IFN-β, IL-6,* and interferon-stimulating genes (ISGs) such as *OAS*, *PKR*, and *MX*, was significantly downregulated in the presence of

minocycline as compared to that without treatment at 48 and 72 h.p.i. (Fig. 10B-E). Although the expression of *IFN-α* significantly increased in minocycline treated groups as compared to that in non-treatment group (Fig. 4A), the relative expression in minocycline-treated groups was less than two-fold as compared with that in the non-treatment group.

DISCUSSION

While minocycline has been used as an antibacterial agent, it has also been reported to exert anti-apoptotic and anti-inflammatory functions [67]. As DTMUV causes severe neurological damage in ducks and that there is no effective treatment against DTMUV, the effects of minocycline on primary duck neurons were analyzed in this study. The present study indicated that minocycline at concentrations up to 40 μg/ml did not cause any cytotoxic effects on DNs. In addition, minocycline protected DNs from death induced by DTMUV infection at 48 and 72 h.p.i. in a dose-dependent manner. Minocycline did not decrease DTMUV replication in treated DNs. Therefore, this study suggests that minocycline exhibits neuroprotective functions against DTMUV infection through anti-apoptotic activity rather than antiviral activity.

Minocycline treatment restored survival rate of DTMUV infected DNs at 48 h.p.i., but its anti-apoptotic effects were observed only at 72 h.p.i. Viral titers were not decreased by minocycline treatment. These results imply that another mechanism rather than apoptosis and viral loads is involved in cell deaths caused by DTMUV. Minocycline has been reported to suppress neuronal cytotoxicity *via* not only anti-apoptotic but also anti-autophagy activity in an animal model of intracerebral hemorrhage [108]. Although autophagy is a cellular pathway involved in cellular homeostasis, excessive autophagy is associated with cancer developments, microorganism infection and several neurodegenerative diseases [30, 65]. Espert *et al.* demonstrated that HIV-1 envelope (Env) protein expression in $CD4^+$ T cell could induced cell death *via* autophagy and inhibition of autophagy totally prevented Env-induced $CD4^+$ T cell death [30]. Previous study demonstrated that DTMUV infection induced autophagy in duck spleen and brain [34]. Based on these results, autophagy might be also involved in cell death caused by DTMUV. This could explain that the survival rates were increased by minocycline treatments at 48 h.p.i. probably because of its anti-autophagic effects. Additional experiments

have to be conducted to reveal the contribution of autophagy to DTMUV pathogenesis and minocycline function.

Minocycline treatment prevented DTMUV-induced cell death but did not decrease the titer of DTMUV. This effect might be associated with the downregulation of *IFN-β* and ISG expression or increased neuronal viability, which was suitable for the second round of viral infection. Similar results have been reported in other viral diseases such as neuroadapted strains of Sindbis virus (NSINV) and rabies virus (RABV) [22, 41, 42]. Minocycline was unable to reduce NSINV replication in mice, despite its neuroprotective effects being enhanced *via* increased astrocyte-mediated glutamate transportation and anti-inflammatory functions [22, 41]. Another study showed that minocycline did not attenuate RABV replication in primary mouse neurons despite its anti-apoptotic functions in infected minocycline-treated mice [42]. This study also showed that minocycline-treated mice had significantly increased cumulative neurological signs and mortality rates as compared to vehicle-treated mice, and decreased migration of $CD3^+$ T cells and reduced neuronal apoptotic cells [42]. Thus, minocycline exerts both positive and negative influence on RABV infection. However, studies have reported that minocycline can also reduce viral titers in some viral diseases [64, 65, 78, 110]. Therefore, minocycline may exhibit different functions depending on the virus. Further studies including *in vivo* experiments are warranted to address this concern in the future. If the increased viral titer induced by minocycline treatment has negative effects on DTMUV-infected birds, minocycline treatment should be carefully adopted by maintaining the balance between its beneficial and deleterious effects on infected birds or performing combination therapy that can compensate the deleterious effects using other drugs such as antiviral agents. Combination treatment with other drugs that have different targets has been tested in patients with ALS and HD [12, 75], and has shown better neuroprotective efficacy in mice than individual drug

treatments [31, 80]. Thus, the combination treatment of minocycline and other antiviral agents should be employed to reduce adverse effects of minocycline in DTMUV-infected birds.

This study presented that minocycline can potentially reduce the upregulation in the expression of proinflammatory cytokines and genes encoding type-I IFN, interleukin-6, and IFN-β. Drews *et al*. indicated that minocycline diminished induction of type I IFN, ISGs, and TNF-related apoptosis inducing ligand (TRAIL) in human plasmacytoid dendritic cells and peripheral blood mononuclear cells after infection with influenza virus or HIV [28]. Previous studies reported that administration of IFN-α/β exacerbate influenza virus-induced apoptosis in murine fibroblast through activation of the Fas-associated death domain-containing protein (FADD)/caspase-8 death signaling pathway [7]. Therefore, decreased mRNA expression of *IFN-β* and ISGs by minocycline treatment might have influence on the levels of apoptosis especially at 72 h.p.i. However, the precise mechanisms underlying minocycline preventing cell death during DTMUV infection need to be investigated.

In addition, the expression of various ISGs, including *OAS*, *PKR*, and *MX*, was downregulated in the present study. This is consistent with the reduction in IFN-β, an activator of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, inducing the transcription of several ISGs [87]. Proinflammatory cytokines play multiple roles in neuropathology of several CNS diseases associated with disease progression [43, 50, 98]. Inhibition of proinflammatory cytokines is thought to be one of effective treatment in CNS diseases [51, 58, 98]. Neuroinflammation is a complex mechanism involving interaction between neurons and non-neuronal cells, including microglia, oligodendrocytes, astrocytes, and immune cells, to regulate inflammatory responses [27, 84]. The present study demonstrated that minocycline treatments (20 and 40 μg/ml) can downregulate the expression of *IL-6* in a dose-dependent manner. Treatment of minocycline might downregulate other proinflammatory genes including *IL-1β*, *IL-8* and tumor necrosis factor *(TNF)-α* in DN-

infected with DTMUV. To understand the detailed mechanisms of an anti-inflammatory activity of minocycline in DTMUV infection, expression levels of other inflammatory genes and proteins needed to be investigated.

In conclusion, these findings demonstrate the neuroprotective effects of minocycline against DTMUV-infected DNs that may be mediated by antiapoptotic functions rather than *via* antiviral functions. This is the first *in vitro* study focusing on drug treatment against DTMUV infection. Our results suggest the plausible application of minocycline to treat DTMUV infection.

Fig. 7.

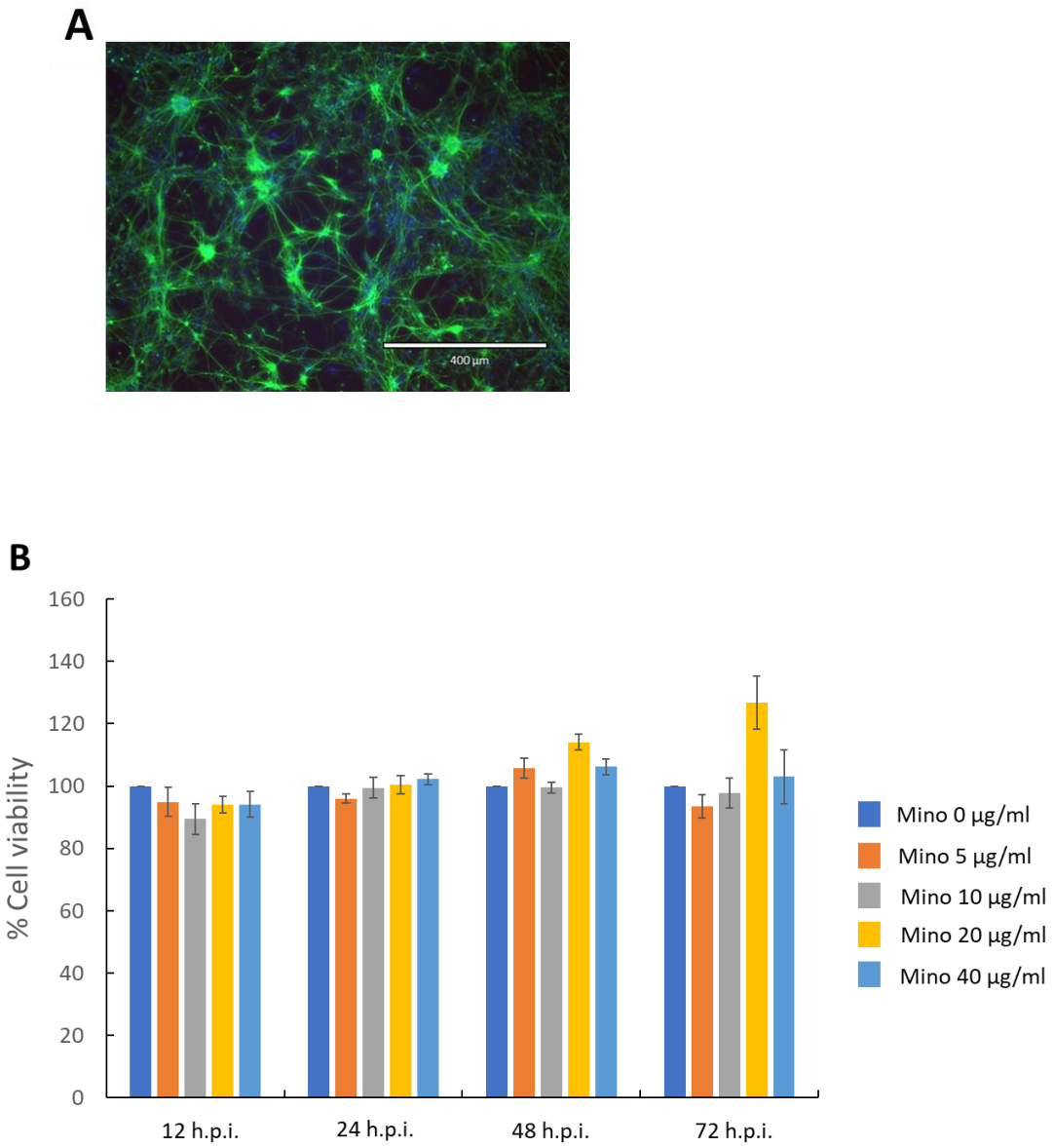

Fig. 7. Morphology of primary duck neurons. (A) An indirect immunofluorescence image of DNs in a culture plate (10×) at 8 days *in vitro* (DIV) (β-III tubulin, neuronal marker: green; and Hoechst 33342, nucleus marker: blue). Scale bar is 400 µm. (B) Viability of minocycline-treated DNs at different time points. Mino indicates minocycline.

Fig. 8.

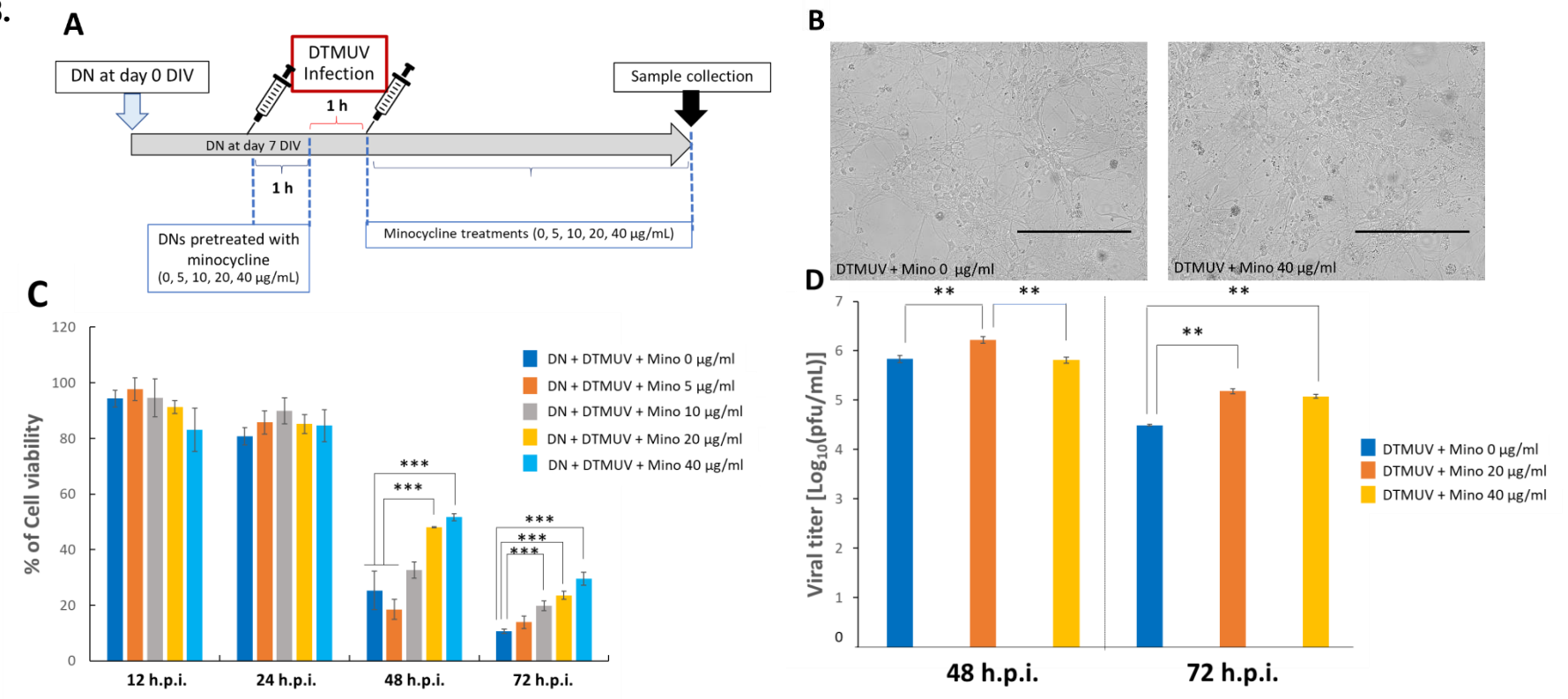

Fig. 8. Effects of minocycline on DTMUV-infected DNs. (A) Schematic experimental plan showing minocycline treatment regimen. (B) Phased-contrast images of DTMUV-infected DNs from non-treatment group and minocycline treatment group (40 µg/ml) at 48 h.p.i. Scale bar is 200 µm. (C) Viabilities of DTMUV-infected and minocycline-treated DNs at indicated time points (12, 24, 48, and 72 h.p.i.). (D) DTMUV titers of minocycline-treated (20 and 40 µg/ml) and non-treated DNs at 48 and 72 h.p.i. The data are represented as the mean ± SEM. Asterisks indicate statistically significant differences among different treatment and non-treatment groups (**P< 0.01; ***P<0.001).

Fig. 9.

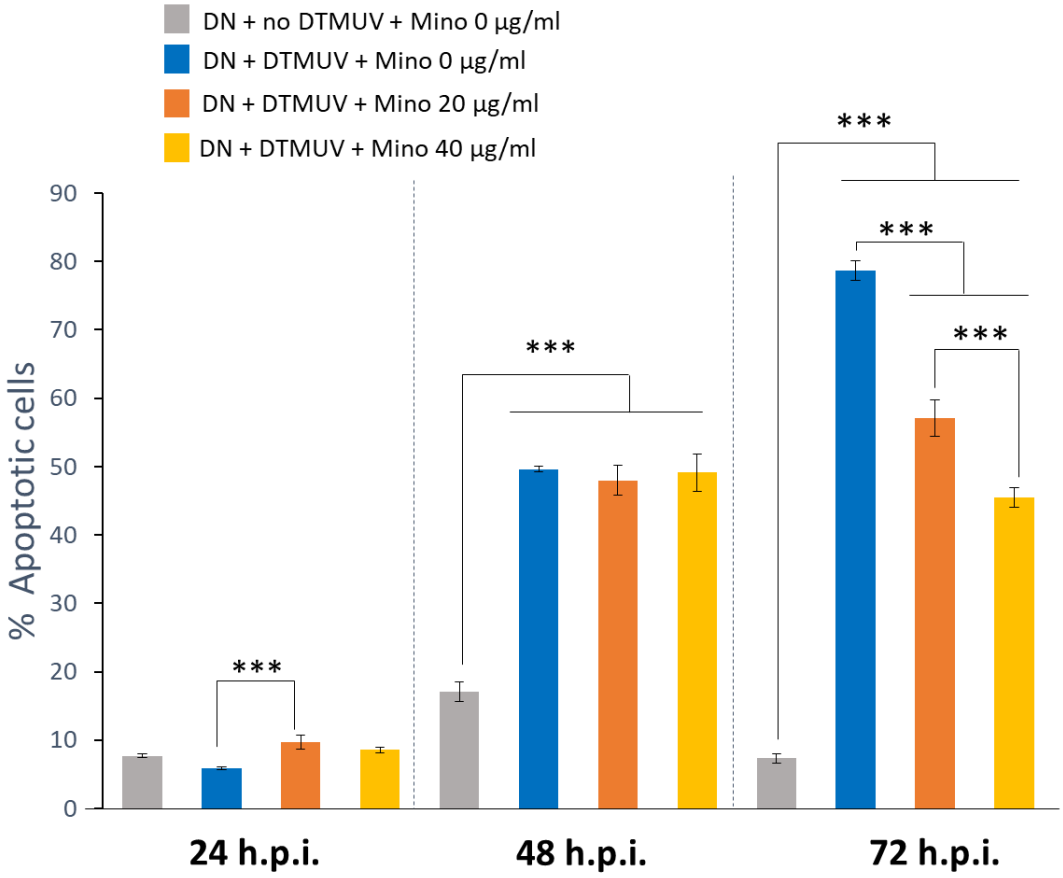

Fig. 9. Minocycline decreased the percentage of apoptotic cells after DTMUV infection. The percentage of apoptotic cells in minocycline-treated and non-treated DNs infected with DTMUV. Data are represented as the mean ± SEM. Asterisks indicate statistically significant differences among different treatment and non-treatment groups (***P< 0.001).

Fig. 10.

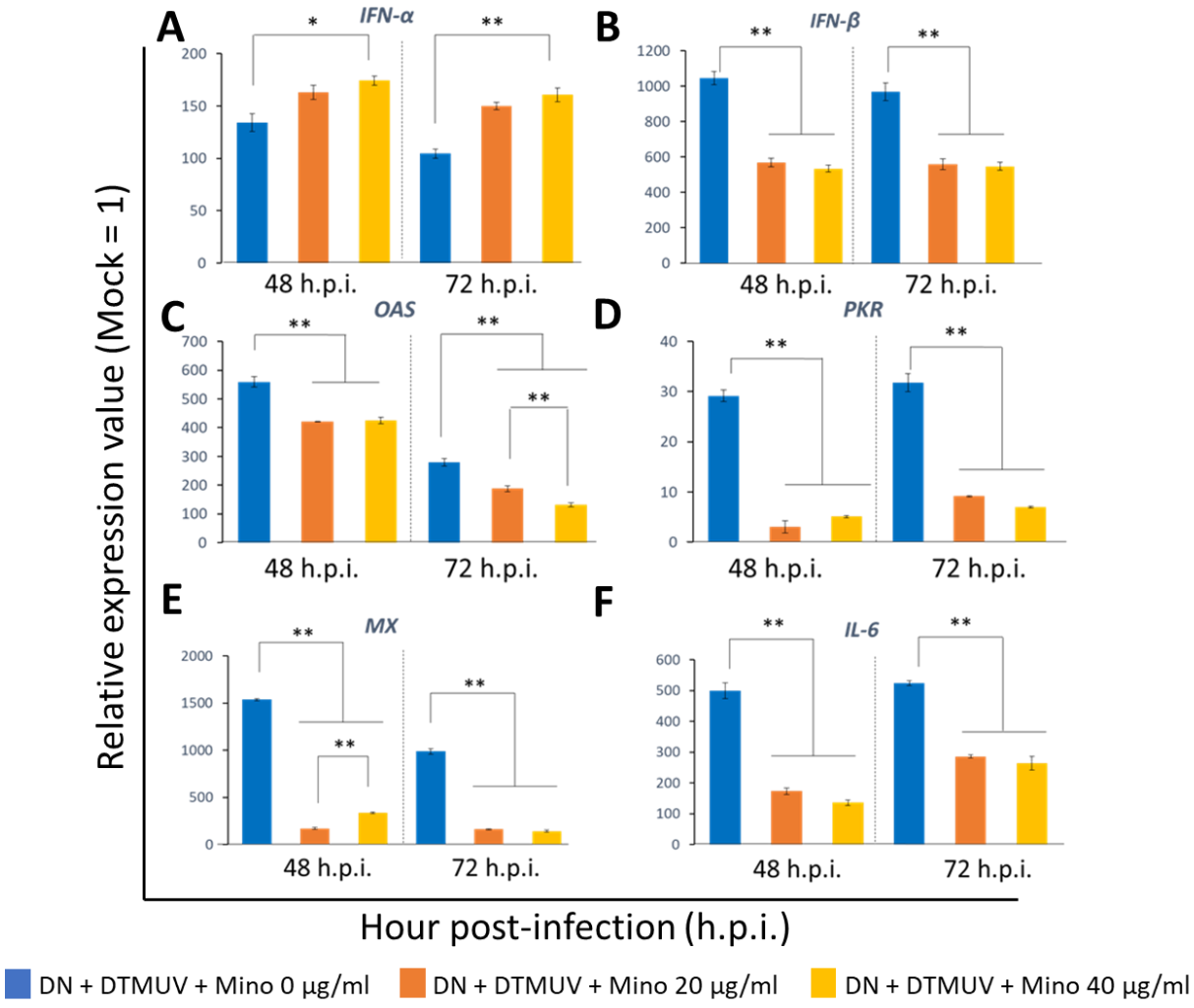

Fig. 10. Expression levels of immune-related genes in DNs with or without minocycline administration. Relative mRNA expression levels of (A) *interferon* (*IFN)-α*, (B) *IFN-β*, (C) *oligoadenylate synthetase (OAS)*, (D) *protein kinase R* (*PKR)*, and (E) *myxovirus resistance (MX*). The results were normalized using the *glyceraldehyde-3-phaosphate dehydrogenase (GAPDH)* expression. The relative expression values were calculated by setting the expression levels in uninfected samples at 48 and 72 h.p.i. as 1 (Mock = 1). Data are represented as the mean ± SEM. Asterisks indicate statistically significant differences among different treatment and non-treatment groups (*P< 0.05; **P< 0.01).

Table 2. Primers used in this study

Primer name	Sequence (5'-3')	Product size (bp)	GenBank no.
IFN-α F	TCCTCCAACACCTCTTCGAC	232	EF053034
IFN-α R	GGGCTGTAGGTGTGGTTCTG		
IFN-β F	AGATGGCTCCCAGCTCTACA	210	KM035791.1
IFN-β R	AGTGGTTGAGCTGGTTGAGG		
IL-6 F	GCAACGACGATAAGGCAGATG	100	AB191038
IL-6 R	TCTTATCCGATTTCAGCTTTGTGA		
MX F	TGCTGTCCTTCATGACTTCG	153	GU202170.1
MX R	GCTTTGCTGAGCCGATTAAC		
OAS F	TCTTCCTCAGCTGCTTCTCC	187	KJ126991.1
OAS R	ACTTCGATGGACTCGCTGTT		
PKR F	AATTCCTTGCCTTTTCATTCAA	109	Unpublished
PKR R	TTTGTTTTGTGCCATATCTTGG		
GAPDH F	ATGTTCGTGATGGGTGTGAA	176	AY436595
GAPDH R	CTGTCTTCGTGTGTGGCTGT		

SUMMARY

Duck Tembusu virus (DTMUV), a neurotropic flavivirus, is a causative agent of severe neurological diseases in different birds. No approved vaccines or antiviral therapeutic treatments are available to date. The poultry industry has experienced significant economic losses due to DTMUV infections. Minocycline is a second-generation semi-synthetic tetracycline analogue that is commonly used as an antimicrobial treatment. Experimental studies have indicated the successful protective effects of minocycline against neuronal cell death from neurodegenerative diseases and viral encephalitis. The aim of this study was to investigate the effects of minocycline on DTMUV infection in neurons. Primary duck neurons were treated with minocycline, which exhibited neuroprotective effects *via* anti-apoptotic function rather than through viral replication inhibition. Minocycline might serve as a potential effective drug in DTMUV infection.

CONCLUSIONS

DTMUV and JEV belong to a mosquito-borne flavivirus. Similar to other neurotropic flaviviruses, DTMUV causes severe neurological signs in various birds. Birds serve as reservoir and amplifier hosts for JEV. In contrast to DTMUV, JEV does not cause noticeable clinical sign in infected birds. Factors responsible for the difference in neuropathogenicity between DTMUV and JEV infection are poorly understood. Due to the lack of suitable avian neuronal cell culture model, mechanisms of virus-induced neuronal cell death as well as neuron-specific innate immune responses in DTMUV- and JEV-infected birds have not been well-investigated. Therefore, the aim of this study was to examine the difference in neuropathogenicity between DTMUV and JEV infection using primary cultured duck neurons (DNs). For the development of possible anti-DTMUV treatment, the efficacy of minocycline treatment in DTMUV-infected DNs was also evaluated.

In Chapter I, the responses of DNs and duck fibroblasts (DFs) to DTMUV KPS54A61 strain and JEV JaGAr-01 strain infection, especially on apoptosis and innate immune responses, were investigated. Both DNs and DFs were highly permissive to DTMUV and JEV infection and replication. DTMUV induced greater degree of caspase-3-dependent apoptosis in DNs and DFs than JEV did. In addition, DTMUV infection induced stronger mRNA expression of several innate immune-related genes in DNs and DFs than JEV infection. However, higher levels of innate immune gene expression observed in DTMUV-infected DNs and DFs did not result in the suppression of viral replication and cell death. The difference in the degree of neuronal apoptosis between DTMUV and JEV infection might be involved in the difference of neuropathogenicity of these viruses in birds. This comparative study provided basic information for understanding the mechanisms of neuropathogenesis in DTMUV and JEV in birds. Also, DNs established in this study will be a valuable and reproducible model for

investigating the mechanisms of various events occur in the neuron of birds infected with DTMUV and JEV.

In Chapter II, the neuroprotective effects of minocycline on DTMUV-infected DNs were investigated, because there was no approved vaccines and effective treatments for DTMUV infection. Minocycline has been reported that have anti-apoptotic and anti-inflammatory properties. It has been broadly used for the treatment of neurological diseases including viral-induced encephalitis. Minocycline protected DNs from cell death induced by DTMUV infection; however, it did not decrease DTMUV replication in treated DNs. These results suggest that minocycline exhibits neuroprotective function against DTMUV infection through the inhibition of apoptosis rather than the inhibition of viral replication.

These studies provide insight on cell death mechanisms and innate immune responses to DTMUV and JEV infection in ducks, and further demonstrate the neuroprotective effects of minocycline on DTMUV infection. Minocycline might be a candidate drug to treat DTMUV infection.

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SUMMARY IN JAPANESE

アヒルテンブスウイルス (DTMUV) と日本脳炎ウイルス (JEV) は共に蚊媒介性フラビウイルスに属する。DTMUV はさまざまな鳥に感染し、他の神経向性フラビウイルスと同様に重度の神経症状を惹き起こす。一方、JEV の生活環において鳥はレゼルボアないし増幅宿主として機能する。DTMUV とは異なり、JEV に感染した鳥は顕著な臨床徴候を示さない。鳥への感染において DTMUV と JEV が示す神経病原性の差異を規定する因子には不明な点が多い。また、鳥類神経細胞の適切な培養モデルがないため、DTMUV および JEV に感染した鳥における神経細胞死と神経細胞に特有な自然免疫応答についてはほとんど研究されていない。本研究は、初代培養アヒル神経細胞 (DN) を用いて DTMUV と JEV の神経病原性を比較検討することを目的とする。抗 DTMUV 治療法の開発の一助となることを目的とし、DTMUV に感染した DN におけるミノサイクリンの有効性も検討した。

第 1 章では、DTMUV KPS54A61 株および JEV JaGAr-01 株の感染により DN とアヒル線維芽細胞 (DF) に誘発される変化をアポトーシスと自然免疫応答に着目して検索した。DN と DF は共に DTMUV と JEV に対し高感受性であり、高レベルのウイルス複製が認められた。DN と DF のいずれにおいても DTMUV は JEV に比較してより強い程度のカスパーゼ 3 依存性アポトーシスを惹起した。さらに、DN と DF のいずれにおいても DTMUV は JEV に比較してより高レベルの自然免疫関連遺伝子発現を誘導した。しかしながら、JEV 感染細胞に比較して DTMUV 感染細胞で観察されたより高レベルの自然免疫関連遺伝子発現はウイルス複製の抑制と細胞死の抑制に寄与していないと考えられた。DTMUV 感染 DN と JEV 感染 DN で認められたアポトーシスの程度の差異は、鳥においてこれらのウイルスが示す神経病原性の差異に関連している可能性が示唆された。これらの比較研究により得られた知見は、鳥における DTMUV と JEV の神経病原性を理解する上で基本的な情報になると考えられる。また、本研究で培養方法が確立された DN は、DTMUV および JEV に感染した鳥の神経細胞で発生するさまざまな現象の機序を解明する上で有益なモデルであると考えられる。

DTMUV 感染症に対する承認されたワクチンや効果的な治療法は存在しない。そこで第 2 章では、DTMUV に感染した DN におけるミノサイクリンの神経保護効果を検討した。ミノサイクリンには抗アポトーシスおよび抗炎症特性があることが報告されており、ウイルス性脳炎を含む種々の神経疾患の治療に広く使用されている。本研究において、ミノサイクリンは DTMUV 感染によって引き起こされる細胞死から DN を保護した。一方で、ミノサイクリン処理された DN において DTMUV 産生量の減少は認められなかった。これらの結果により、ミノサイクリンがウイルス複製の阻害ではなく、アポトーシスの阻害を通じて DTMUV 感染に対する神経保護機能を示すことが示唆された。

以上の研究により、DTMUV および JEV の感染によってアヒルに引き起こされる細胞死のメカニズムと自然免疫応答に関する基礎的知見が提供され、さらに DTMUV 感染に対するミノサイクリンの神経保護効果を明らかになった。ミノサイクリンは DTMUV 感染症の治療において候補薬になる可能性を有する。