



Minocycline prevents primary duck neurons from duck Tembusu virus-induced death

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ABSTRACT. 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 experiences 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.

KEY WORDS: bird, duck Tembusu virus, minocycline, neuroprotective effect, primary duck neuron

J. Vet. Med. Sci.

83(4): 734–741, 2021

doi: 10.1292/jvms.20-0735

Received: 5 January 2021

Accepted: 23 February 2021

Advanced Epub:

15 March 2021

Duck Tembusu virus (DTMUV) is a mosquito-borne flavivirus that is mainly transmitted by *Culex tritaeniorhynchus* mosquitoes [37, 43]. It is a causative agent of severe neurological diseases in various bird species, including ducks, geese, chickens, pigeons, and sparrows [15, 25, 41, 42, 47]. Laying and breeding ducks show a sudden decline in egg production following infection with DTMUV [39]. Clinical signs of reproductive problems have been documented as hyperemic and flaccid ovaries and oophoritis [27, 32, 39]. Affected birds showed severe neurological symptoms such as unbalanced gait, wing and leg paralysis, and head tremor [32]. Histopathological analysis confirmed encephalitis comprising moderate to severe lymphocytic inflammation surrounding the perivascular spaces and diffuse glia nodules [27]. Further, a high titer of DTMUV was reported in the central nervous system (CNS) organs of infected ducks [27, 32]. Hence, DTMUV is known as a neurotropic flavivirus and continuous to emerge and reemerge in several countries, including China, Malaysia, and Thailand [14, 31, 44]. However, no approved vaccines or therapeutic treatments are yet available.

Apoptosis normally occurs during tissue development, homeostasis of adult tissues, and defense mechanisms [10]. However, inappropriate apoptosis is commonly associated with diseases such as cancer and neurodegenerative disorder [10]. Several neurotropic viruses induce neuronal death via apoptosis, which plays a pivotal role in neuropathogenesis, concurrent with uncontrolled inflammatory responses [5]. Our preliminary study also indicates that DTMUV can induce apoptosis in primary duck neurons (DNs), which is correlated with a reduction of cell viability after DTMUV infection (unpublished data).

Minocycline is a second-generation semi-synthetic tetracycline derivative that is well-known as a bacteriostatic antibiotic which effectively crosses the blood-brain barrier [33]. 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 [13, 34, 35, 48].

Minocycline inhibits replication of several neurotropic viruses such as the Japanese encephalitis virus (JEV), West Nile virus (WNV), dengue virus (DENV), Simian immunodeficiency virus (SIV), and human immunodeficiency virus (HIV) [3, 28, 30, 49]. An experimental study on JEV infection demonstrated significant decrease in viral replication, proinflammatory mediators, and neuronal apoptosis in minocycline-treated mice [29, 30]. 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 [28]. Further, WNV-infected spinal cord slice cultures (SCSC) treated with minocycline showed decreased viral replication, proinflammatory microglial activation, and apoptosis in neurons [35]. Minocycline also exerted antiviral effects in mouse neuroblastoma Neuro2a (N2a) infected with four different serotypes of DENV (DENV 1–4), although it failed to reduce the number of dead cells [23].

Neurological dysfunctions prevent DTMUV-infected birds from normal activity and get them emaciated. This makes them

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more susceptible to other viral and bacterial infections, resulting in high morbidity and mortality [45]. Minocycline has been preliminarily used in human clinical trials to treat neurological diseases based on the successful results in several experimental studies. Several studies have confirmed successful outcomes with minocycline in spinal cord injury, multiple sclerosis, and JEV infection [22, 33]. Considering the usefulness of minocycline for neurological diseases, we hypothesized that minocycline treatment may also work for neurological symptoms caused by DTMUV. Moreover, we thought that minocycline could also reduce reproductive problems caused by DTMUV. Previous histological studies suggest that mononuclear cell infiltration in ovarian interstitium may play a role in the destruction of ovarian follicles of DTMUV-infected birds [4, 32, 39]. Therefore, anti-inflammatory function of minocycline might have a potential to decrease pathological changes in reproductive organs of DTMUV-infected birds by repressing inflammatory responses.

Previous findings encouraged us to investigate the effects of minocycline on DTMUV infection. We examined the effects of minocycline on DTMUV-infected primary duck neurons (DNs) and the underlying mechanism of action. 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. Our culture protocol was modified from the long-term primary chicken neuron culture method, as previously described [21]. 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 solution (Merck Millipore, Billerica, MA, USA). Duck neurons were cultured at 37°C, and the old medium was replaced with fresh medium supplemented with 1 μM cytosine β-D-arabinofuranoside (Ara-C; Sigma-Aldrich, St. Louis, MO, USA). After 6 to 7 days *in vitro* (DIV), 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. Taweesak Songserm at the Faculty of Veterinary Medicine, Kasetsart University (Nakhon Pathom, Thailand) [6].

Immunofluorescence assay (IFA)

For neuronal cell detection, DNs at 6 or 7 DIV 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 hr. 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 hr. 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) (Fig. 1A).

Viral titration assay

The DTMUV titer was determined by a plaque assay using BHK cells. Supernatants were collected from infected plates at 48 hr post-infection (hr p.i.). In brief, 70% to 80% confluent BHK cells were infected with DTMUV for 1 hr. The plate was washed with PBS and overlaid with MEM supplemented with 5% FBS and 1.25% methyl cellulose. After 72 hr 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). 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 DIV were pretreated with minocycline at different concentrations (0, 5, 10, 20, and 40 μg/ml) for 1 hr, and infected with DTMUV at a multiplicity of infection (MOI) of 1 for 1 hr. 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. 2A. The plates were incubated at 37°C in a CO₂ 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

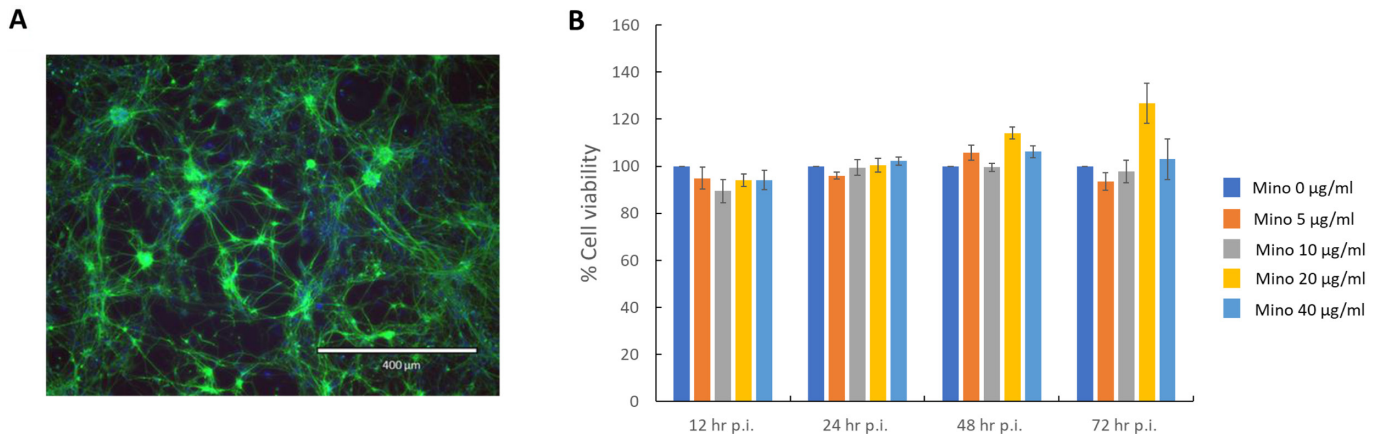


Fig. 1. Morphology of primary duck neurons (DNs). (A) An indirect immunofluorescence image of DN in a culture plate (10 \times) at 8 day *in vitro* (DIV) (β -III tubulin, neuronal marker: green; and Hoechst 33342, nucleus marker: blue). Scale bar is 400 μ m. (B) Viability of minocycline-treated DN at different time points.

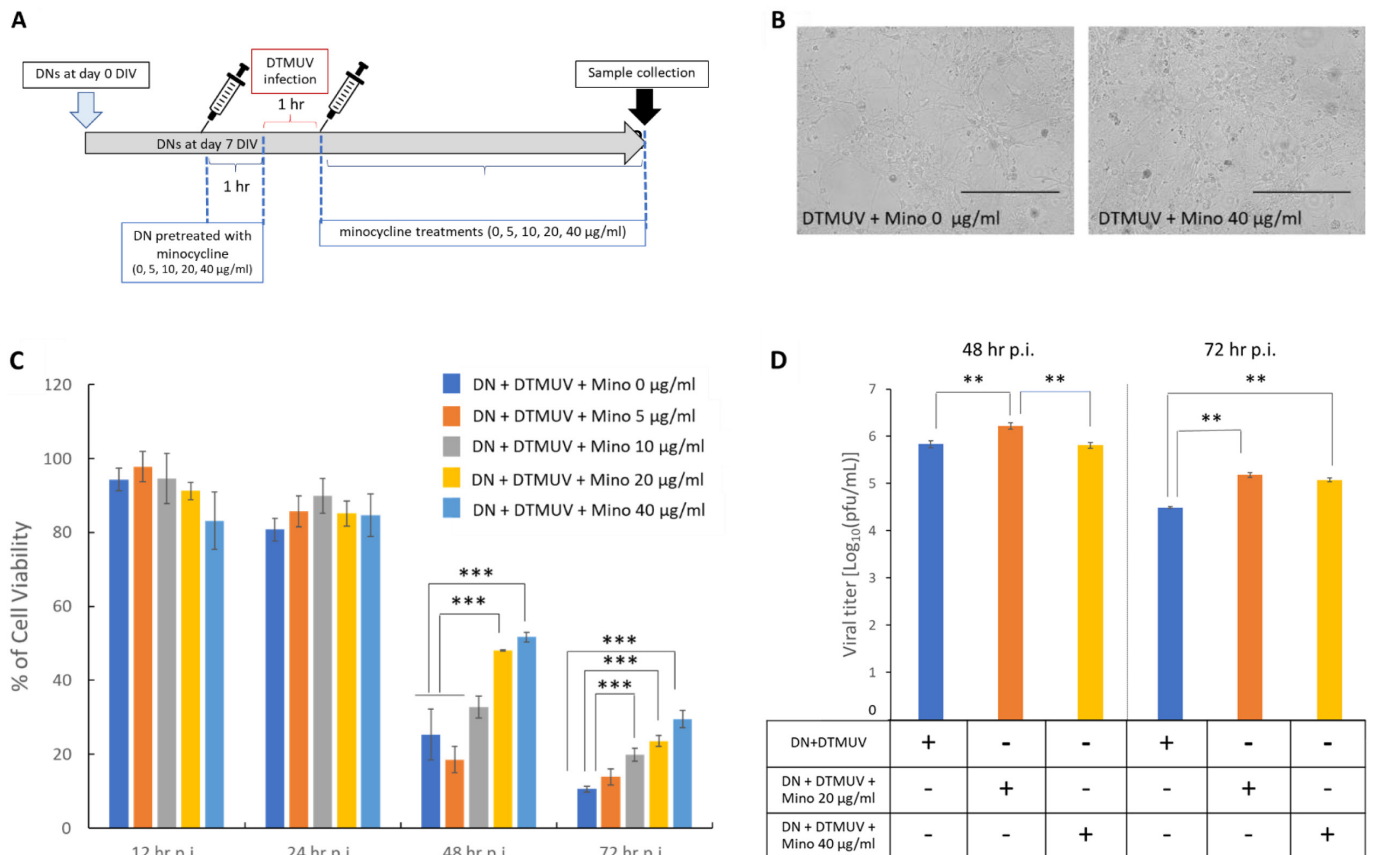


Fig. 2. Effects of minocycline on duck Tembusu virus (DTMUV)-infected primary duck neurons (DNs). (A) Schematic experimental plan showing minocycline treatment regimen. (B) Phased-contrast images of DTMUV-infected DN from non-treatment group and minocycline treatment group (40 μ g/ml) at 48 hr p.i. Scale bar is 200 μ m. (C) Viabilities of DTMUV-infected and minocycline-treated DN at indicated time points (12, 24, 48, and 72 hr p.i.). (D) DTMUV titers of minocycline-treated (20 and 40 μ g/ml) and non-treated DN at 48 and 72 hr p.i. The data are represented as the mean \pm SEM. Asterisks indicate statistically significant differences among different treatment and non-treatment groups (** P <0.01; *** P <0.001).

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 hr p.i.) was calculated. Next, 10 μ l of CCK-8 reagent was added to each well, and the cells were incubated in the dark at 37°C for 2 hr. The absorbance was measured at 450 nm wavelength with a Nanodrop™ (Thermo Fisher Scientific).

Flowcytometry 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 hr 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, USA) was used to analyze the data.

RNA isolation and cDNA preparation

At 48 and 72 hr 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, Kusatsu, Japan), and cDNA was synthesized using PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio) according to the manufacturer's protocols.

Real-time reverse-transcription quantitative polymerase chain reaction (qPCR)

The qPCR 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. qPCR 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 endogenous amplification control for data normalization. Finally, melting curve analysis was carried out to confirm the specificity of the primers. The primers used for qPCR are listed in Table 1 [1, 7, 24].

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. 1A). To investigate the cytotoxic effects of minocycline, DNs were treated with various concentrations of minocycline (0, 5, 10, 20, and 40 μ g/ml) and their viability was analyzed at several time points (12, 24, 48, and 72 hr p.i.). The results showed that minocycline treatment had no effect on DN viability over the course of 72 hr (Fig. 1B).

Next, to examine the effective minocycline dose that protected DNs from death induced by DTMUV infection, we infected DNs

Table 1. 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	TCTTATCCGATTTTCAGCTTTGTGA		
MX F	TGCTGTCCTTCATGACTTCG	153	GU202170.1
MX R	GCTTTGCTGAGCCGATTAAC		
OAS F	TCTTCCTCAGCTGCTTCTCC	187	KJ126991.1
OAS R	ACTTCGATGGACTCGCTGTT		
PKR F	AATTCCTTGCCCTTTTCATTCAA	109	Unpublished
PKR R	TTTGTTTTGTGCCATATCTTGG		
GAPDH F	ATGTTTCGTGATGGGTGTGAA	176	AY436595
GAPDH R	CTGTCTTCGTGTGTGGCTGT		

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; MX, myxovirus resistance; OAS, oligoadenylate synthetase; PKR, protein kinase R.

with DTMUV (MOI=1) following treatment with different concentrations of minocycline. The cells were pretreated with minocycline for 1 hr, infected with DTMUV for 1 hr without minocycline, and then cultured for 12, 24, 48, and 72 hr p.i. with minocycline (Fig. 2A). In comparison with the untreated cells, DTMUV-infected DNs showed morphology similar to DTMUV-infected DNs with 40 µg/ml minocycline treatment at 48 hr p.i. (Fig. 2B); however, minocycline treatment increased cell viability in a dose-dependent manner (Fig. 2C). Although there was no effect of minocycline on DN viability at 12 and 24 hr p.i. at all concentrations, 20 and 40 µg/ml minocycline concentrations significantly increased cell viability at 48 and 72 hr p.i. and 10 µg/ml minocycline treatment showed a significant increase in cell viability at 72 hr p.i. (Fig. 2C).

To investigate the effect of minocycline on DTMUV replication, we compared DTMUV titers between minocycline treatment groups (20 and 40 µg/ml) and non-treatment group at 48 and 72 hr 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 hr p.i. (Fig. 2D).

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 hr 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. 3). At 48 hr p.i., the proportion of apoptotic cells was comparable between minocycline-treated and non-treated DTMUV-infected DNs. However, at 72 hr 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 works in a dose-dependent manner, consistent with the survival rate of cells observed in the cell viability assay (Fig. 2C). 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, we examined the expression levels of inflammatory genes in DTMUV-infected DNs that were untreated or treated with 20 and 40 µg/ml minocycline at 48 and 72 hr p.i. using qPCR. At the mRNA level, the expression of *interferon (IFN)-β*, *interleukin (IL)-6*, and *interferon-stimulating genes (ISGs)* such as *oligoadenylate synthetase (OAS)*, *protein kinase R (PKR)*, and *myxovirus resistance (MX)*, was significantly downregulated in the presence of minocycline as compared to that without treatment at 48 and 72 hr p.i. (Fig. 4B–E). Although the expression of *IFN-α* significantly increased in minocycline treatment 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. As DTMUV causes severe neurological damage in ducks and that there is no effective treatment against DTMUV, here we analyzed the effects of minocycline on primary duck neurons. We found 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 hr p.i. in a dose-dependent manner. Minocycline did not decrease DTMUV replication in treated DN. Therefore, we suggest 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 hr p.i., but its anti-apoptotic effects were observed only at 72 hr 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 [48]. Although, autophagy

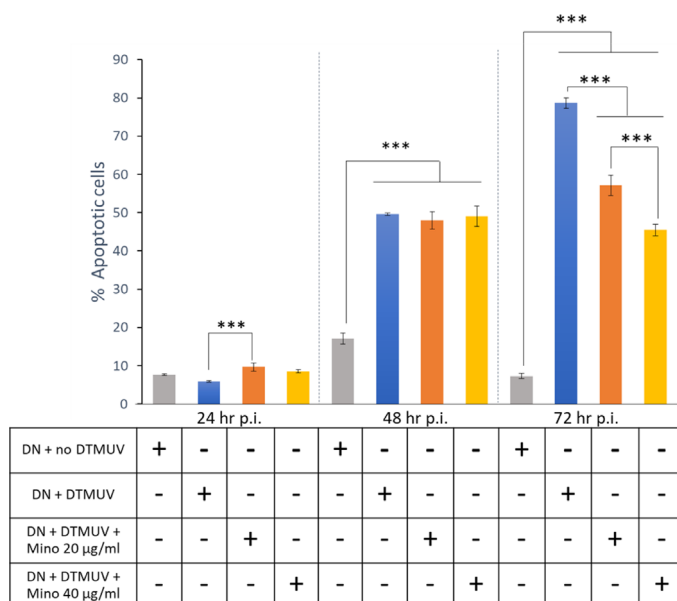


Fig. 3. Minocycline decreased the percentage of apoptotic cells after duck Tembusu virus (DTMUV) infection. The percentage of apoptotic cells in minocycline-treated and non-treated primary duck neurons (DNs) infected with DTMUV. Data are represented as the mean ± SEM. Asterisks indicate statistically significant differences among different treatment and non-treatment groups (***) $P < 0.001$.

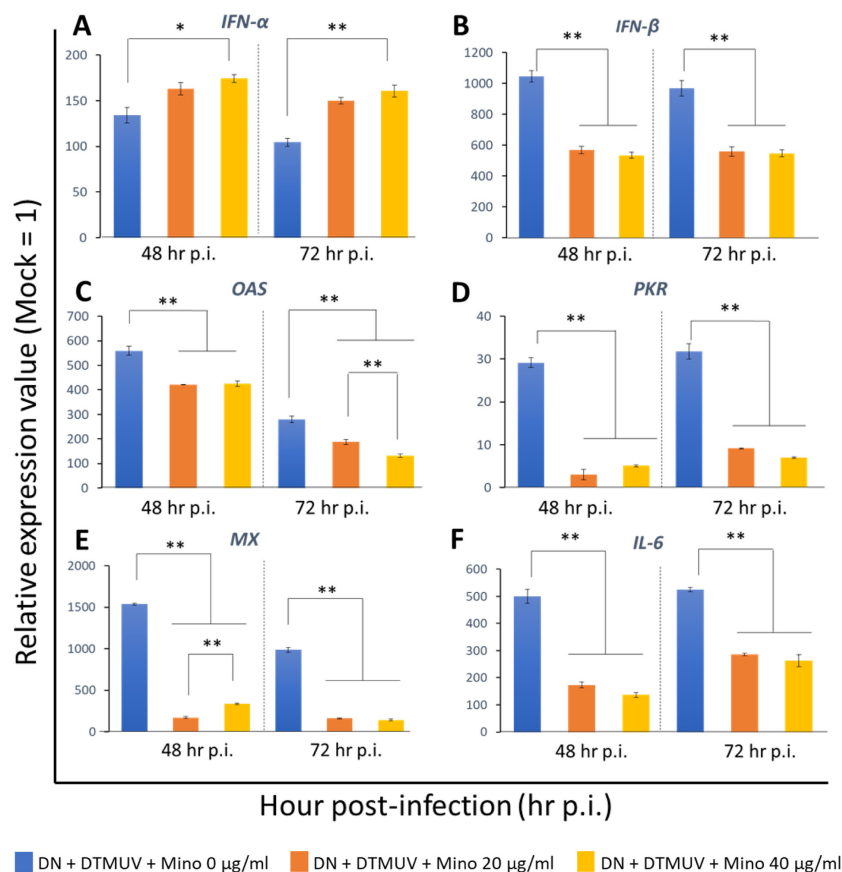


Fig. 4. Expression levels of immune-related genes in primary duck neurons (DNs) with or without minocycline administration. Relative mRNA expression levels of (A) *interferon (IFN)-α*, (B) *IFN-β*, (C) *oligoadenylate synthetase (OAS)*, (D) *protein kinase R (PKR)*, and (E) *myxovirus resistance (MX)*. The results were normalized using the *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* expression. The relative expression values were calculated by setting the expression levels in uninfected samples at 48 and 72 hr 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 (* $P < 0.05$; ** $P < 0.01$).

is a cellular pathway involved in cellular homeostasis, excessive autophagy is associated with cancer developments, microorganism infection and several neurodegenerative diseases [30]. 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 [11]. Previous study demonstrated that DTMUV infection induced autophagy in duck spleen and brain [13]. 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 hr 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 neuro-adapted Sindbis virus (NSV) and rabies virus (RABV) [8, 16, 17]. Minocycline was unable to reduce NSV replication in mice, despite its neuroprotective effects being enhanced via increased astrocyte-mediated glutamate transportation and anti-inflammatory functions [8, 16]. Another study showed that minocycline did not attenuate RABV replication in primary mouse neurons despite its anti-apoptotic functions in infected minocycline-treated mice [17]. 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 [17]. 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 [29, 30, 35, 49]. 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 [2, 33], and has shown better neuroprotective efficacy in mice than individual drug treatments [12, 36]. Thus, the combination treatment of minocycline and other antiviral agents should be employed to reduce adverse effects of minocycline in DTMUV-infected birds.

We found that minocycline can potentially reduce the upregulation in the expression of proinflammatory cytokines and genes encoding type-I interferon (IFN), interleukin-6, and *IFN-β*. In addition, the expression of various ISGs, including *OAS*, *PKR*, and *MX*, was downregulated. 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 [40]. Proinflammatory cytokines play multiple roles in neuropathology of several CNS diseases associated with disease progression [18, 19, 46]. Inhibition of

proinflammatory cytokines is thought to be one of effective treatment in CNS diseases [20, 26, 46]. Neuroinflammation is a complex mechanism involving interaction between neurons and non-neuronal cells, including microglia, oligodendrocytes, astrocytes, and immune cells, to regulate inflammatory responses [9, 38]. In the present study, we 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, we demonstrate the neuroprotective effects of minocycline against DTMUV-infected DN 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.

CONFLICT OF INTERESTS. The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS. We would like to thank Prof. Taweesak Songserm, Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand, for providing DTMUV-KPS54A61. This work was supported by Kasetsart Veterinary Development Funds.

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