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APOPTOSIS STUDY OF INDONESIAN AVIAN INFLUENZA VIRUS SUBTYPE H5N1 IN MADIN-DARBY CANINE KIDNEY CELLS

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ABSTRACT

This study aimed to determine the ability of highly pathogenic avian influenza virus (HPAI) virus subtype H5N1 originated from Indonesia to induce apoptosis in Madin-Darby Canine Kidney (MDCK) cells. Three HPAI virus subtype H5N1 isolates with different genetic characteristic namely A/Bird/Bali1/2011, A/Chicken/East Java/BwiI2/2010 and A/Chicken/West Java/1074/2003, were cultured in MDCK cells. Apoptosis was identified by deoxyribonucleic acid (DNA) fragmentation of infected MDCK cells using Apoptotic DNA Ladder Kit. The results showed that all three HPAI virus isolates used in this study did not able to induce apoptosis in the MDCK cells within 5 to 72 hours post infection.

Key words: apoptosis, influenza virus subtype H5N1, MDCK cells

ABSTRAK

Penelitian ini bertujuan mengetahui kemampuan virus highly pathogenic avian influenza virus *(HPAI) subtipe H5N1 asal Indonesia dalam menginduksi apotosis pada sel* Madin-Darby Canine Kidney *(MDCK). Percobaan dilakukan pada tiga isolat virus yang berbeda karakter genetik yaitu A/Bird/Bali1/2011, A/Chicken/East Java/BwiI2/2010 dan A/Chicken/West Java/1074/2003 yang diinfeksikan pada sel MDCK. Pengujian apoptosis dilakukan dengan melihat fragmentasi* deoxyribonucleic acid *(DNA) sel MDCK yang diinfeksi virus dengan menggunakan kit Apoptotic DNA Ladder. Hasil penelitian menunjukkan bahwa ketiga virus yang digunakan dalam penelitian ini tidak mampu memicu terjadinya apoptosis pada sel MDCK dalam rentang waktu inkubasi 5-72 jam.*

Kata kunci: apoptosis, virus influenza subtipe H5N1, sel MDCK

INTRODUCTION

Influenza virus has caused serious illness both in human and animals even with death consequence (Murphy and Webster, 1990). This virus infects epithelial cell of respiratory tract causing cell death. There are two factors that morphologically and biochemically responsible for cell death namely necrosis and apoptosis. Necrosis is a pathological reaction occurred as a response of disturbances, such as hypoxia, hypothermia, etc. Such inducements increase plasma membrane permeability, which can cause irreversible cell swelling. In contrast, apoptosis or programmed cell death is a physiological process in normal tissues occurred in several cases such as embryogenesis, aging, or in the presence of tumor (Cheung *et al*., 2002). Apoptosis is marked by nucleus condensation and cell fragmentation into apoptotic bodies that can be rapidly ingested by macrophages or neighbor cells (Steller, 1995). Apoptosis may occur as a response triggered by toxin promoting or inhibiting hormones and cytokines (Hinshaw *et al*., 1994). Moreover, apoptosis also often happens in pathological processes, for example cancer cells, inflammation cells, infected microbe cell, abnormal cell metabolism, and others (Thompson, 1995).

In the case of microbial infection, several of viruses have been proved causing apoptosis to host cells (Teodoro and Branton, 1997). Furthermore, several strains of influenza A including highly pathogenic avian influenza (HPAI) have been reported inducing apoptosis in various type of cells as *in vitro* studies as well as *in vivo* studies (Takizawa *et al*., 1993; Fesq *et al*., 1994; Hinshaw *et al*., 1994; Mori *et al*., 1995; Clarke and Tyler, 2009). Although the mechanism and function of apoptosis during influenza A virus infection have been not clearly understood yet, it is suspected as a cellular anti-viral mechanism to limit virus replication.

Influenza A viruses have developed several ways to delay apoptosis as the host defense strategies (Kurokawa *et al*., 1999; Zhirnov *et al*., 2002; Ehrhardt *et al*., 2007; Zhirnov and Klenk, 2007). On the other hand, there are several influenza viruses that induce apoptosis via pro-apoptotic factors in order to increase propagation efficiency. Several viral proteins such as NA and PB1-F2 contain pro-apoptotic function (Palese and Shaw, 2007). Temporal regulation of pro- and anti-apoptotic mechanism is crucial for virus pathogenesis. Controlling apoptosis during initial infection is regulated instantly so virus has sufficient time to replicate in host cells. Mean while, apoptosis induction at the end of infection will release virus progenies. Apoptosis occur after virus replication will increase phagocytic cleansing of infected cells, which may induce cell mediated cytotoxic response.

So far, there is no report related to apoptotic information for HPAI virus subtype H5N1 from Indonesia. The H5N1 virus has already killed more than 100 people in Indonesia. Thus, the Indonesian H5N1 viruses have been assumed having different characters compared to other viruses from different regions. The main objective of this study is to investigate whether HPAI virus subtype H5N1 from Indonesia can induce apoptosis upon infection in Madin-Darby Canine Kidney (MDCK) cells. The MDCK cells was chosen due to its high sensitivity against HPAI virus compared with other cells such as the green monkey continuous cell line (Vero) or human lung embryonated cells (MRC-5) (Reina *et al*., 1997; Govorkova *et al*., 1999).

MATERIALS AND METHODS

Highly Pathogenic Avian Influenza Subtype H5N1 Viruses

The HPAI virus subtype H5N1 isolates used in this study have been already characterized in the previous studies, namely A/Bird/Balil/2011, A/Chicken/East Java/Bwil2/2010 and A/Chicken/West Java/1074/2003 (Dharmayanti *et al*., 2011; Dharmayanti *et al*., 2014). These viruses were propagated in 9-11 days old embryo of specific pathogen free (SPF) chicken eggs. These viruses were chosen based on its genetic characteristic difference. The A/Bird/Bali1/2011 virus was isolated from the vicinity of human cases of H5N1 virus infection. The A/Chicken/East Java/Bwil2/2010 virus displays genetic drift mutation. Then, the A/Chicken/ West Java/1074/2003 virus is typical H5N1 virus isolated at the beginning of HPAI outbreak in Indonesia (Dharmayanti *et al*., 2004).

Madin-Darby Canine Kidney (MDCK) Cells

The MDCK cells in cryogenic ampoules stored frozen in liquid nitrogen were thawed at 37° C and then centrifuged at speed of 1,000-1,500 rpm for 5 minutes until sedimentation appeared at the bottom of the ampoules. Then, cell sediment was diluted using Dulbecco's Modified Eagle's Medium (DMEM) and re-cultivated in the T25 flask (NUNC). Incubation was done in an incubator at temperature 37° C with 5% concentration of CO_2 and relative humidity (RH) around 95%. The MDCK cell development was observed daily until confluent monolayer was formed. The MDCK cells were ready for the next passage or to be used for virus inoculation.

Virus Infection in Madin-Darby Canine Kidney (MDCK) Cells

The MDCK cells were cultivated in the 6 wells plate (NUNC) until confluent monolayer was formed. The media was discarded with only small amount was leftover. As much as 100 µL of the respective avian influenza virus isolate was inoculated into the MDCK cells. The virus absorption into the MDCK cells was done by incubation at 37° C for 30 minutes. Then, in about 5 ml of DMEM was transferred into plate tissue culture and re-incubated at 37° C. Cell growth and condition were observed in designated period times post infection by microscope, which are 5 hours, 24 hours, 36 hours, 48 hours, and 72 hours. The MDCK cells with respective times post infection for the three avian influenza viruses were stored at -20° C for the next analysis.

DNA Extraction of Infected Madin-Darby Canine Kidney (MDCK) Cells

The deoxyribonucleic acid (DNA) extraction for apoptotic test was done using Apoptotic DNA Ladder Kit (Roche). Briefly, in about 200 µL of MDCK cell sample infected by HPAI viruses from each observation times were taken into a sterile 1.5 mL microcentrifuge tube. It was added with 200 µL of PBS and 200 µL of binding/lysis buffer, and then homogenized before incubation at 15° C - 25° C for 10 minutes. Later, 100 µL of isopropanol was added and then homogenized again. Subsequently, the mixture was transferred into tube assembled with filter. Centrifugation was done at speed of 8,000 rpm for 1 minute. Collection tube containing filtrate was discarded and replaced by new collection tube. About 500 µL of buffer (blue cap) was added into filter tube and centrifuged at 8,000 rpm for 1 minute. Centrifugation was done again at 13,000 rpm for 10 seconds. Collection tube was discarded, while filter tube was inserted into a new sterile 1.5 mL microcentrifuge tube. The DNA elusion was done by adding 200 µL of elution buffers into filter tube. Centrifugation was done at 8,000 rpm for 1 minute. The DNA suspension of each treatment was stored at -20° C for the next analysis.

Apoptosis Observation Through Electrophoresis

Electrophoresis of DNA was done in the 2% Agarose gel with TBE 1x buffer. As much as 2 µL of loading dye was mixed with 5 µL DNA samples and then injected into agarose gel wells. In addition, ladder DNA 100 bp (Invitrogen) was used as marker. Electrophoresis was run with 100 volts for 30 minutes. The profile of DNA was visualized using UV transilluminator apparatus. Apoptosis of cells is marked by DNA fragmentation. If the fragmented bands were observed, the cell is positive for apoptosis.

RESULTS AND DISCUSSION

Apoptosis evaluation in this study used Apoptotic DNA Ladder Kit (Roche). Positive control of apoptosis from cell showed many band of DNA strips (Figure 1). On the other hand, the MDCK cells infected with Bali1/11, BwiI2/10, and West Java/1074/03 viruses did not show any incidence of apoptosis (Figure 1). Similarly, the MDCK cells infected by Bali1/11, BwiI2/10, and West Java/1074/03 viruses also did not show any sign of apoptosis after 24 hours (Figure 2), 36 hours (Figure 3), and 48 hours (Figure 4) post-infection.

At 36, 48, and 72 hours post infection (Figure 3, 4 and 5), apoptosis was not detected in MDCK cells infected by A/bird/Bali/Bangli 1/2011(H5N1), A/Chicken/ East Java/BwiI2/2010(H5N1) and A/Chicken/West Java/1074/2003(H5N1) viruses marked by the lack of fragmented bands. There were cell destructions in MDCK cell infected by the viruses; however, it was not caused by apoptosis. This damage was marked by denaturation bands, which did not point to any base position. In this matter, uninfected MDCK cells did not show any cell destruction. Interpretation of DNA fragmentation profile in MDCK cells infected by avian influenza H5N1 virus showed that three virus isolates used in this research did not induce apoptosis (Table 1). The observation indicated that MDCK cell infected by H5N1 virus may experience necrosis.

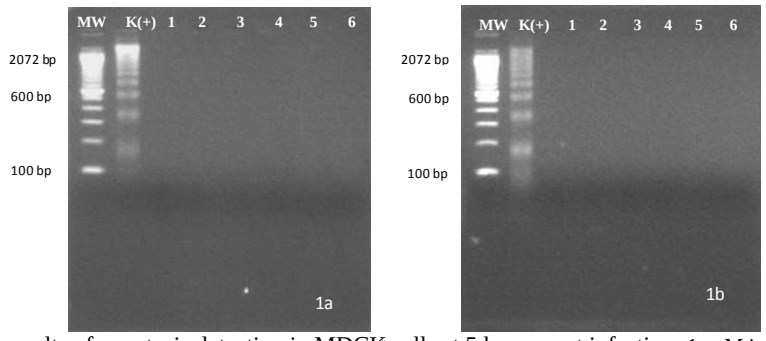

Figure 1. Electrophoresis results of apoptosis detection in MDCK cells at 5 hours post infection. 1a= M is 100 bp DNA Marker, K(+) is apoptosis positive control, well 1 and 2 are MDCK cells infected by Bali1/11 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells. 1b= Well 1 and 2 are MDCK cells infected by BwiI2/10 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells.

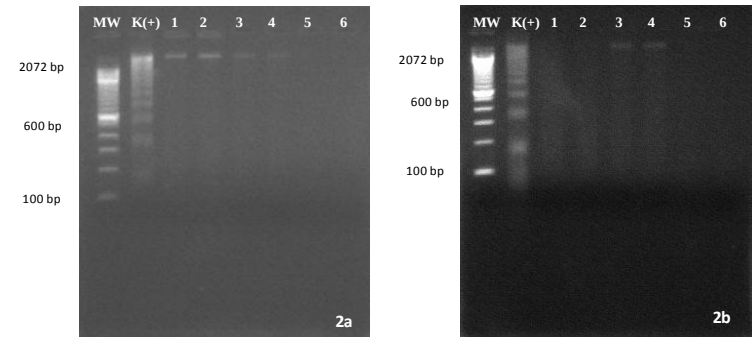

Figure 2. Electrophoresis result of apoptosis detection in MDCK cell at 24 hours post infection. 2a= M is 100 bp DNA Marker, K(+) is apoptosis positive control, well 1 and 2 are MDCK cells infected by Bali1/11 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells. 2b= Well 1 and 2 are MDCK cells infected by BwiI2/10 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells.

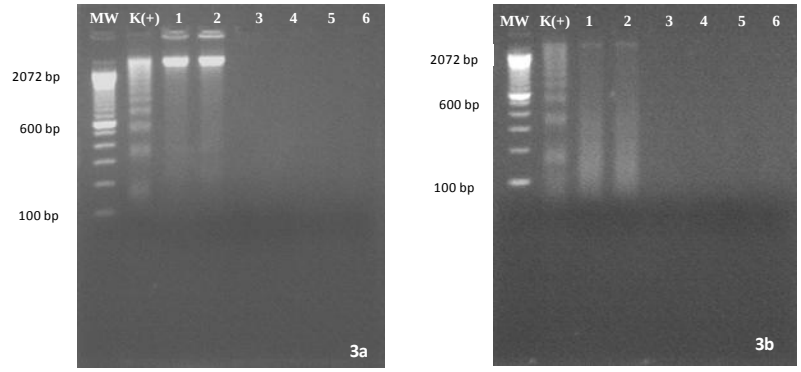

Figure 3. Electrophoresis result of apoptosis detection in MDCK cell in 36 hours post infection. 3a= M is 100 bp DNA Marker, K(+) is apoptosis positive control, well 1 and 2 are MDCK cells infected by Bali1/11 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells. 3b= Well 1 and 2 are MDCK cells infected by BwiI2/10 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells.

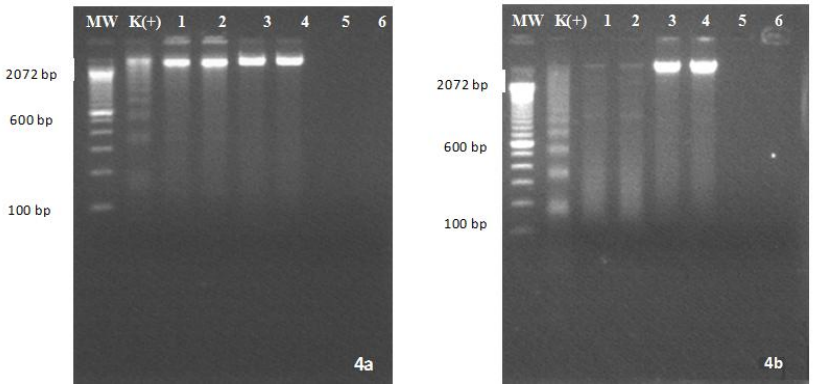

Figure 4. Electrophoresis result of apoptosis detection in MDCK cell in 48 hours post infection. 4a= M is 100 bp DNA Marker, K(+) is apoptosis positive control, well 1 and 2 are MDCK cells infected by Bali1/11 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells. (4b) Well 1 and 2 are MDCK cells infected by BwiI2/10 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells.

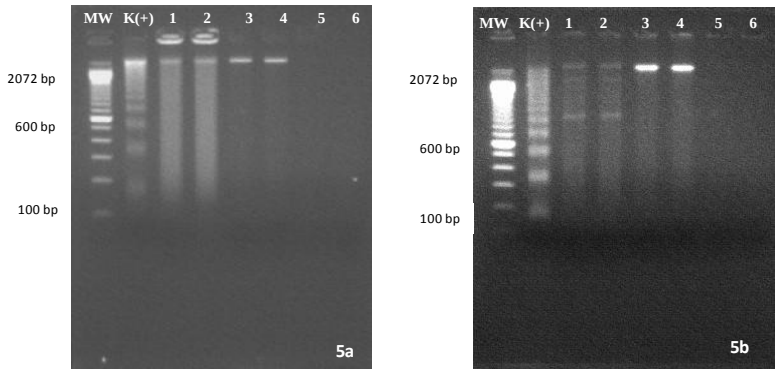

Figure 5. Electrophoresis result of apoptosis detection in MDCK cell in 72 hours post infection. 5a= M is 100 bp DNA Marker, K(+) is apoptosis positive control, well 1 and 2 are MDCK cells infected by Bali1/11 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells. 5b= Well 1 and 2 are MDCK cells infected by BwiI2/10 virus, well 3 and 4 are MDCK cells infected by West Java/1074/03 virus, well 5 and 6 are uninfected MDCK cells.

Table 1. Apoptosis detection based on DNA fragmentation profile in MDCK cells

Hours post infection	Sample Identity	Result of apoptosis detection
5	A/Bird/Bali1/2011 replicate 1	negative
	A/Bird/Bali1/2011 replicate 2	negative
	A/Chicken/West Java/1074/2003 replicate 1	negative
	A/Chicken/West Java/1074/2003 replicate 2	negative
	A/Chicken/East Java/BwiI2/2010 replicate 1	negative
	A/Chicken/East Java/BwiI2/2010 replicate 2	negative
	uninfected cell control replicate 1	negative
	uninfected cell control replicate 2	negative
	positive control	positive
24	A/Bird/Bali1/2011 replicate 1	negative
	A/Bird/Bali1/2011 replicate 2	negative
	A/ Chicken /West Java/1074/2003 replicate 1	negative
	A/Chicken/West Java/1074/2003 replicate 2	negative
	A/Chicken/East Java/BwiI2/2010 replicate 1	negative
	A/Chicken/East Java/BwiI2/2010 replicate 2	negative
	uninfected cell control replicate 1	negative
	uninfected cell control replicate 2	negative
	positive control	positive
36	A/Bird/Bali1/2011 replicate 1	negative (denaturation)
	A/Bird/Bali1/2011 replicate 2	negative (denaturation)
	A/ Chicken /West Java/1074/2003 replicate 1	negative
	A/ Chicken /West Java/1074/2003 replicate 2	negative
	A/Chicken/East Java/BwiI2/2010 replicate 1	negative (denaturation)
	A/Chicken/East Java/BwiI2/2010 replicate 2	negative (denaturation)
	uninfected cell control replicate 1	negative
	uninfected cell control replicate 2	negative
	positive control	positive
48	A/Bird/Bali1/2011 replicate 1	negative (denaturation)
	A/Bird/Bali1/2011 replicate 2	negative (denaturation)
	A/ Chicken /West Java/1074/2003 replicate 1	negative (denaturation)
	A/ Chicken /West Java/1074/2003 replicate 2	negative (denaturation)
	A/Chicken/East Java/BwiI2/2010 replicate 1	negative (denaturation)
	A/Chicken/East Java/BwiI2/2010 replicate 2	negative (denaturation)
	uninfected cell control replicate 1	negative
	uninfected cell control replicate 2	negative
	positive control	positive
72	A/Bird/Bali1/2011 replicate 1	negative (denaturation)
	A/Bird/Bali1/2011 replicate 2	negative (denaturation)
	A/ Chicken /West Java/1074/2003 replicate 1	negative (denaturation)
	A/ Chicken /West Java/1074/2003 replicate 2	negative (denaturation)
	A/Chicken/East Java/BwiI2/2010 replicate 1	negative (denaturation)
	A/Chicken/East Java/BwiI2/2010 replicate 2	negative (denaturation)
	uninfected cell control replicate 1	negative
	uninfected cell control replicate 2	negative
	positive control	positive

Several studies reported that influenza A and B viruses could induce apoptosis, a programmed cell death mechanism as a cellular antiviral mechanism that restrict virus replication (Takizawa *et al*., 1993; Hinshaw *et al*., 1994; Uiprasertkul *et al.,* 2007). In apoptosis cell, morphological changes take place in the form of fragmented nucleus development, chromatin condensation and loss of cell volume. In many cases, apoptosis requires protein synthesis followed by influx of Ca^{2+} within the cytosol. Ca^{2+} may cause chromosome DNA fragmented into oligonucleosome (Cheung *et al*., 2002). Takizawa *et al.* (1993) mentioned that influenza virus infection could cause chromosome DNA fragmentation into oligonucleosome led to nucleus fragmentation and chromatin condensation. In the case of influenza virus infection, the process is inhibited by protein synthesis inhibitor (Cheung *et al*., 2002).

One of virus genes assumed to be responsible in apoptosis mechanism is NS1. However, the role of NS1 in apoptosis has not been completely elucidated yet since NS1 gene has both pro- and anti-apoptosis properties (Schultz-Cherry *et al*., 2001; Zhirnov *et al*., 2002; Stasakova *et al*., 2005; Ehrhardt *et al.,* 2007; Shin *et al.,* 2007a; Lam *et al.,* 2008). The antagonistic function NS1 gene might be consequence of the specific experimental protocols such as type of cells or strains of virus. Interestingly, NS1 gene is hypothesized contributing temporarily on stress at the beginning of apoptosis giving slow induction to cell death.

Study by Zhirnov *et al*. (2002) in influenza virus strain PR8 infection showed that anti-apoptotic function of NS1 gene is associated with its ability to limit production and effect of interferon (IFN). In competent MDCK cells, the IFN of PR8 delNS1 mutant virus having deletion in NS gene induce apoptosis higher than wild type PR8 virus (Zhirnov *et al*., 2002). However, in Vero cells these two viruses induced apoptosis at the same rate even slower than in MDCK cells (Zhirnov *et al*., 2002). It is not known if Vero cell is also deficient in other genes beside IFNα/β. It is assumed that IFNα/β antagonist NS1 is the most important factor in limiting apoptosis. Catalytic active PKR is also reported having a role in apoptosis during influenza virus infection (Takizawa *et al*., 1993). Direct binding and inhibition of PKR by NS1 can also produce stress towards cell death. The same thing might be happened in inhibition mediated by NS1, which is OAS/RNase L pro-apoptosis (Min and Krug, 2006) or JNK/AP-1 pathway (Ludwig *et al*., 2002). In addition, PI3K host cell pathway activation was described as additional mechanism of NS1 possibly limiting apoptosis induction (Ehrhardt *et al.,* 2007; Shin *et al.,* 2007b; Zhirnov and Klenk, 2007).

In this study, the cell destruction caused by virus replication facilitates to easier release and faster spread of virus to nearby cells. It was observed in MDCK cell infected by A/bird/Bali1/2011 and A/Chicken/East Java/BwiI2/2010 virus that cell damage occurred started from 36 hours post infection. It is 12 hours faster than the infection of A/Ck/West Java/1074/2003. Apoptosis is known to be the host cell's defense mechanism which limits virus replication and eliminates virus from the host. Several studies reported that numerous viruses have mechanism to halt apoptosis so virus able to replicate efficiently before apoptosis is finished or make use of the apoptosis mechanism itself (Teodoro and Branton, 1997; Ludwig *et al.,* 2003; Ehrhardt and Ludwig, 2009). The method used in the current research cannot show the apoptosis of H5N1 virus originating from Indonesia in MDCK cell. However, Takizawa *et al*. (1993) stated that H5N1 virus with low replication ability might exhibit apoptosis. This incidence showed that virus replication indirectly causing cell to lyse. The research of Daidoji *et al*. (2008) reported that H5N1 virus could induce apoptosis in epithelial mammalian airways cells.

The lack of apoptosis or observed apoptosis incidence in this study might be caused by the strains used in this research. These H5N1 viruses maybe able overcome the apoptosis. Other explanation is that the viruses replicate too intensively so the cells underwent necrosis before apoptosis process finished. The result of this research showed that the characteristic avian influenza virus in inducing apoptosis is highly dependent on virus strains and type of cells as well as incubation time of infection. Furthermore, further studies with different virus strains and methodologies are required to investigate the apoptosis phenomenon of HPAI viruses subtype H5N1 from Indonesia.

CONCLUSION

The result of this study showed that three HPAI viruses subtype H5N1 from Indonesia with different genetic characteristic, namely A/Bird/Bali1/2011, A/Chicken/East Java/BwiI2/2010, and A/Chicken/West Java/1074/2003, did not able to induce apoptosis in the MDCK cell infection within 5-72 hours post infection.

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REFERENCES

Cheung, C.Y., L.L. Poon, A.S. Lau, W. Luk, Y.L. Lau, K.F. Shortridge, S. Gordon, Y. Guan, and J.S.M. Peiris. 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: A mechanism for the unusual severity of human disease? **Lancet**. 360:1831-1837.

Clarke. P. and K.L. Tyler. 2009. Apoptosis in animal models of virus-induced disease. **Nat. Rev. Microbiol.** 7:144-155.

Daidoji, T., T. Koma, A. Du, C.S. Yang, M. Ueda, K. Ikuta, and T. Nakaya. 2008. H5N1 avian influenza virus induces apoptotic cell death in mammalian airways epithelial cells. **J. Virol.** 82:11294-11307.

Dharmayanti, N.L.P.I., G. Samaan, F. Ibrahim, R. Indriani, Darminto, and A. Soebandrio. 2011. The Genetic Drift of Indonesian Avian

Influenza A H5N1 Viruses During 2003-2008. **Microbiol. Indones.** 5:68-80

Dharmayanti, N.L.P.I., A. Ratnawati, D.A. Hewajuli, and R. Indriani. 2014. Genetic characterization of H5N1 avian influenza viruses isolated from pet bird and chickens from live bird market in Bali and Bekasi (Indonesia). **AJMR**. 8:244-251.

Dharmayanti, N.L.P.I., R. Damayanti, A. Wiyono, R. Indriani, and Darminto. 2004. Identification of avian influenza virus of Indonesian isolates by reverse transcripatese polymerase chain reaction RT-PCR (RT-PCR) method. **IJAVS.** 9:136-143.

Ehrhardt, C. and S. Ludwig. 2009. A new player in a deadly game: Influenza viruses and the PI3K/Akt signalling pathway. **Cell Microbiol.** 11:863-871.

Ehrhardt, C., T. Wolff, S. Pleschka, O. Planz, W. Beermann, J.G. Bode, M. Schmolke, and S. Ludwig. 2007. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. **J. Virol.** 81:3058-3067.

Fesq, H., M. Bacher, M. Nain, and D. Gemsa. 1994. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. **Immunobiology.** 190:175-82.

Govorkova, E.A., S. Kodihalli, I.V. Alymova, B. Fanget, and R.G. Webster. 1999. Growth and immunogenicity of influenza viruses cultivated in vero or MDCK cells and in embryonated chicken eggs. **Dev. Biol. Stand**. 98:39-51.

Hinshaw, V.S., C.W. Olsen, N. Dybdahl-Sissoko, and D. Evans. 1994. Apoptosis: A mechanism of cell killing by influenza A and B viruses. **J. Virol.** 68:3667-3673.

Kurokawa, M., A.H. Koyama, S. Yasuoka, and A. Adachi. 1999. Influenza virus overcomes apoptosis by rapid multiplication. **Int. J. Mol. Med.** 3:527-530.

Lam, W.Y., J.W. Tang, A.C. Yeung, L.C. Chiu, J.J. Sung, and P.K. Chan. 2008. Avian influenza virus A/HK/483/97(H5N1) NS1 protein induces apoptosis in human airway epithelial cells. **J. Virol.** 82:2741-2751.

Ludwig, S., O. Planz, S. Pleschka, and T. Wolff. 2003. Influenza-virus-induced signaling cascades: targets for antiviral therapy? **Trends Mol. Med.** 9:46-52.

Ludwig, S., X. Wang, C. Ehrhardt, H. Zheng, N. Donelan, O. Planz, S. Pleschka, A. Garcia-Sastre, G. Heins, and T. Wolff. 2002. The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors. **J. Virol.** 76:11166-11171.

Min, J.Y. and R.M. Krug. 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. **Proc. Natl. Acad. Sci. USA.** 103:7100-7105.

Mori, I., T. Komatsu, K. Takeuchi, K. Nakakuki, M. Sudo, and Y. Kimura. 1995. In vivo induction of apoptosis by influenza virus. **J. Gen. Virol.** 76:2869-73.

Murphy, B.R. and R.G. Webster. 1990. Orthomyxoviruses. In Virology 2nd ed. Edited by B.N. Fields and D.M. Knipe. Raven Press, New York.

Palese, P. and M.L. Shaw. 2007. Orthomyxoviridae: The Viruses and Their Replication. In **Fields Virology**. Knipe, D.M. and P.M. Howley (Eds.). 5th ed. Lippincott Williams and Wilkins, Philadelphia.

Reina, J., V. Fernandez-Baca, I. Blanco, and M. Munar. 1997. Comparison of madin-darby canine kidney cells (MDCK) with a green monkey continuous cell line (Vero) and human lung embryonated cells (MRC-5) in the isolation of influenza a virus from nasopharyngeal aspirates by shell vial culture. **J. Clin. Microbiol**. 35 (7):1900-1901.

Schultz-Cherry, S., N. Dybdahl-Sissoko, G. Neumann, Y. Kawaoka, and V.S. Hinshaw. 2001. Influenza virus NS1 protein induces apoptosis in cultured cells. **J. Virol.** 75:7875-7881.

Shin, Y.K, Q. Liu, S.K. Tikoo, L.A. Babiuk, and Y. Zhou. 2007b. Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. **J. Gen. Virol.** 88:13-18.

Shin, Y.K., Y. Li, Q. Liu, D.H. Anderson, L.A. Babiuk, and Y. Zhou. 2007a. SH3 binding motif 1 in influenza A virus NS1 protein is essential for PI3K/Akt signalling pathway activation. **J. Virol.** 81:12730-12739.

Stasakova, J., B. Ferko, C. Kittel, S. Sereinig, J. Romanova, H. Katinger, and A. Egorov. 2005. Influenza A mutant viruses with altered NS1 protein function provoke caspase-1 activation in primary human macrophages, resulting in fast apoptosis and release of high levels of interleukins 1b and 18. **J. Gen. Virol.** 86:185-195.

Steller, H. 1995. Mechanisms and genes of cellular suicide. **Science.** 267:1445-1449.

Takizawa, T., S. Matsukawa, Y. Higuchi, S. Nakamura, Y. Nakanishi, and R. Fukuda. 1993. Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. **J. Gen. Virol.** 74:2347-2355.

Teodoro, J.G. and P.E. Branton. 1997. Regulation of apoptosis by viral gene products. **J. Virol.** 71:1739-1746.

Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. **Science.** 267:1456-1462.

Uiprasertkul, M., R. Kitphati, P. Puthavathana, R. Kriwong, A. Kongchanagul, K. Ungchusak, S. Angkasekwinai, K. Chokephaibulkit, K. Srisook, N. Vanprapar, and P. Auewarakul. 2007. Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. **EID**.13:708-712.

Zhirnov, O.P. and H.D. Klenk. 2007. Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. **Apoptosis**. 12:1419-1432.

Zhirnov, O.P., T.E. Konakova, T. Wolff, and H.D. Klenk. 2002. NS1 protein of influenza A virus down-regulates apoptosis. **J. Virol.** 76:1617-1625.