CHAPTER VI
DISSCUSSION

Neurological complications including encephalitis have been reported in several mosquito-born disease cases. Japanese encephalitis virus (JEV) infection is a major cause of acute encephalopathy especially in children in Southeast Asia [25]. Recently, there are increased evidences of encephalitis in patients with Dengue infection around the world. In 2009, WHO released new dengue guidelines that consider CNS complications in the clinical case as severe dengue [55]. However, mechanism involved in dengue encephalitis has not been elucidated. In case of JE encephalitis, microglia, the residential macrophages in the brain, have been proposed as the most important player that may act as "Trojan horse" for virus infiltration to CNS [69], and also release several factors and cytokines that finally resulting in neuronal death [57, 58].

Morphological of microglia cells in resting stage are in the round shape. They transform to amoeboid phagocytic shape in response to a stimulus such as LPS and JEV. The present study, transformation of HAPI cells was an evidence for microglia activation after challenging with JEV, DENV-2 and DENV-4 as well as CxFV. The activated microglia have changed from round-shape to ameboi form [59].

Reactive oxygen species (ROS) have been hypothesized to contribute in viral pathogenesis, viral replication, host defense and modulation of cellular responses [54]. The present study demonstrated the elevation of intracellular ROS production in HAPI cells after challenging with all viruses. The results suggested that ROS may cause oxidative stress and thus resulted in reduction of cell viability. It should be noted that at first 24 hours ROS production was about 1.2-1.4 fold comparing with mock control, and after 24 hours ROS production was increased about 1.6-2.0 fold. According to this result, it may be proposed that there were two phases of ROS production, the first phase was rapid response due to the virus and the second phase may be a consequence of oxidative stress produced in the cells.

There is no clearly explanation related to viral-induced ROS production in the cells. The binding of growth factor and cytokine to their receptor trigger the rapid intracellular ROS production, especially superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) which play important role in the signal transduction [60]. ROS have been implicated in activation of nuclear factor kappa B (NF-kB) which plays a central role for expression of inducible nitric oxide synthase (iNOS) [61]. Here, the effect of viruses on activation of NF-kB was not investigated. However, the results clearly showed the enhanced ROS production and iNOS protein expression in HAPI cells after challenging with JEV, CxFV and DENV-2. In case of JEV, enhanced iNOS expression in relation to ROS and NF-kB has been demonstrated in several cells systems including neuroblastoma, glia and macrophages as well as in mice models. Since nitric oxide (NO) inhibits RNA virus replication, NO is known as antiviral molecule. Thus, enhanced iNOS could be a target for treatment of JEV and others virus infection.

Although DENV-2 and CxFV revealed the increased iNOS expression, the pathway of iNOS induction may not be same as JEV. In some of viral diseases, iNOS expression is regulated via interferon-γ (IFN-γ) and in some cases viral components per se directly induced iNOS [62]. Interestingly, DENV-4 did not induce iNOS expression. Li Q and Engelhardt JF have reported that high concentration H_2O_2 inhibited NF-κB activity. It may postulate that high ROS production in DENV-4, particularly at 6 hours pi, may inversely inhibit iNOS in DENV-4. To proof this hypothesis, activity of NF-κB needs to be determined.

Beside iNOS expression, level of NO production is important to determine the biological effects of NO [5, 7]. Physiologic levels (nano molar) function as a signal transduction molecule whereas the higher levels act as a toxic molecule [5,6]. The toxicity of NO result from the reaction of NO with $O_2^{\cdot -}$, yielding peroxynitrite ($ONOO^-$). Depending on the concentration (and disease situation), NO acts as anti-inflammatory and also pro-inflammatory [67]. The present study found that release of NO (detected as nitrate) to culture medium was high as micro molar (1-2 μM). Corresponding with iNOS expression, JEV seemed to produce higher concentration of NO. On the other hand, NO production was undetectable in DENV-4. In order to

clarify the role of NO in flaviviral infection, precise quantitation of NO and qualification of ROS are necessary.

ROS are known as common mediators for apoptosis [63]. In this present study, expression and activation of caspase-3 was examined. Nazmi A et al., 2011 have been reported that JEV infected macrophages activated caspase-3, induced Bax and suppressed Bcl2 protein expression [64]. In this study, JEV and CxFV slightly increased caspase-3 expression (1.2 fold) and activated caspase-3 whereas those were not changed in DENV-2. Interestingly, activation of caspase-3 was suppressed in DENV-4. In contrast with the result of cell viability, DENV-4 markedly decreased cell viability. The results may suggest that decreased in cell viability in DENV-4 may implicate by others pathways including anti-apoptotic processes. Moreover, there are many pathways for cell growth and cell proliferation are redox sensitive [65]. In addition, ROS also induces cell death by necrosis process. These results called an attention to study the factors/pathways that involve in the distinctive outcomes of two DENV serotypes.

As mention earlier, there might be two phases of ROS production. Viruses *per se* may induced the rapid production of ROS and thus trigger the signaling pathways such as NF-kB. The second phase could be a consequence of oxidative products such as NO release, cytokine release and other components from damaged cells [66]. This hypothesis may support by our other separate experiments that ROS production was sharply decreased after replacing fresh medium to the culture system (i.e. at 24 hours post infection).

It should be noted that the results related to virus infection in cell culture systems are largely depend on several factors including virus strain, cell type, concentration of virus (MOI) and period of study time. Although the present study used low MOI (0.1) of viruses comparing with other reports (MOI 0.5-30), this concentration was sufficient to demonstrate microglia responses including cell activation, enhanced ROS production and iNOS expression. In reality, amount of transmitted virus may not be such high as the study *in vitro*.