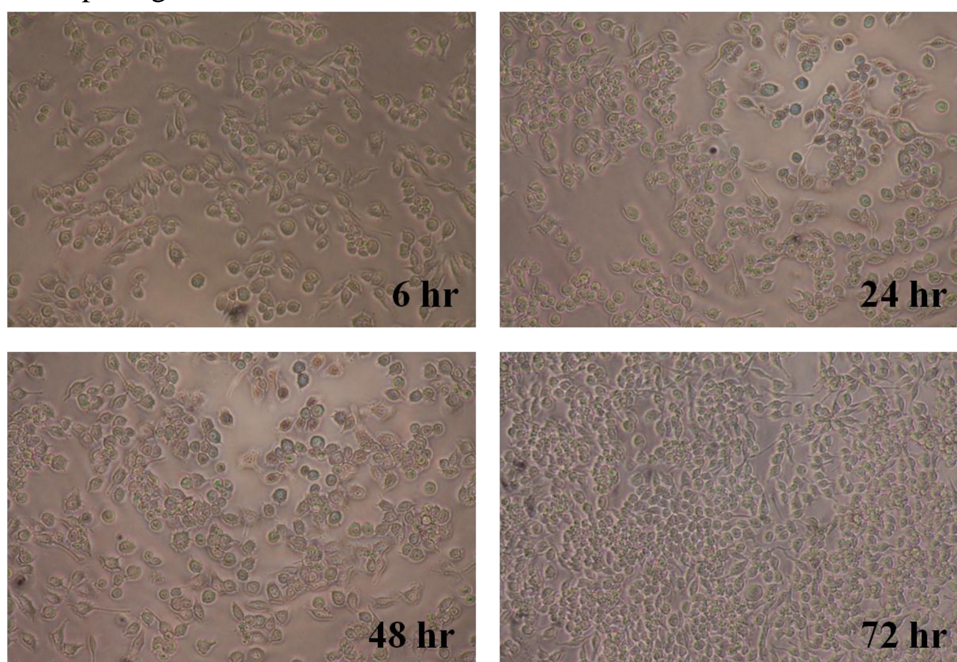
CHAPTER V
RESULTS

5.1 Characteristic of HAPI cells and cell response after LPS and t-Butyl hydroperoxide (t-BuOOH) activation.

In resting stage, HAPI cells are typically in rounded shape without differentiation. Morphology of HAPI cells at 6, 24, 48 and 72 hrs after plating in 96 well were observed and demonstrated in Figure 5.1. Cell viability of various time presented in Figure 5.2

In all experiments, HAPI cell at the passage 40-43 was used. Figure 5.3 indicated that there was no significant difference in the cell viability profile of the studied cell passages.



10X

Figure 5.1 Morphological of HAPI cells growth under normal condition.
HAPI cell at density of $1x10^4$ cells/well on 96 well plates were cultured in medium supplemented with 2.5% FBS and observed the morphology at 6, 24, 48 and 72 hours. The images were taken at 10X original magnification.

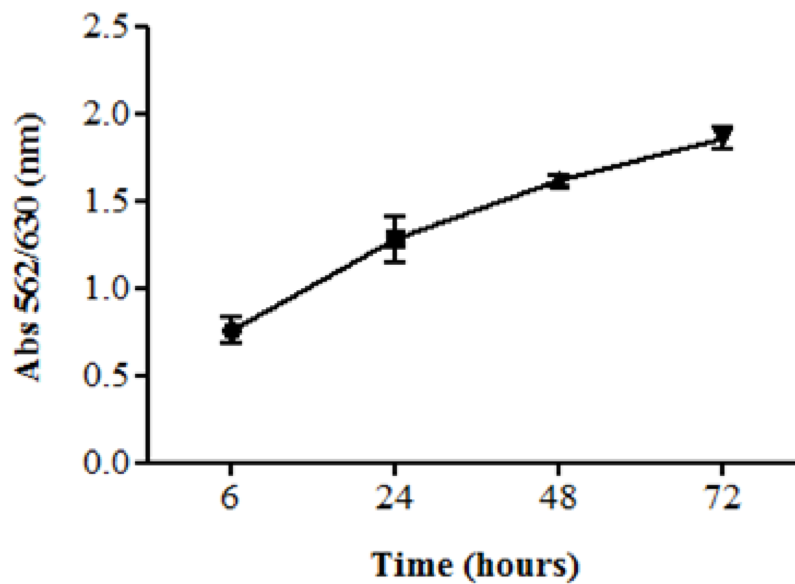


Figure 5.2 Cell viability of HAPI cells growth under the control condition.

HAPI cells at density 1×10^4 cells/well on 96 well plates were cultured in DMEM supplemented with 2.5% FBS under control condition. Absorbance of formazan crystal (wavelength 562 and 630 nm) was measured at 6, 24, 48 and 72 hours. Data are presented as mean ± SEM (n=8).

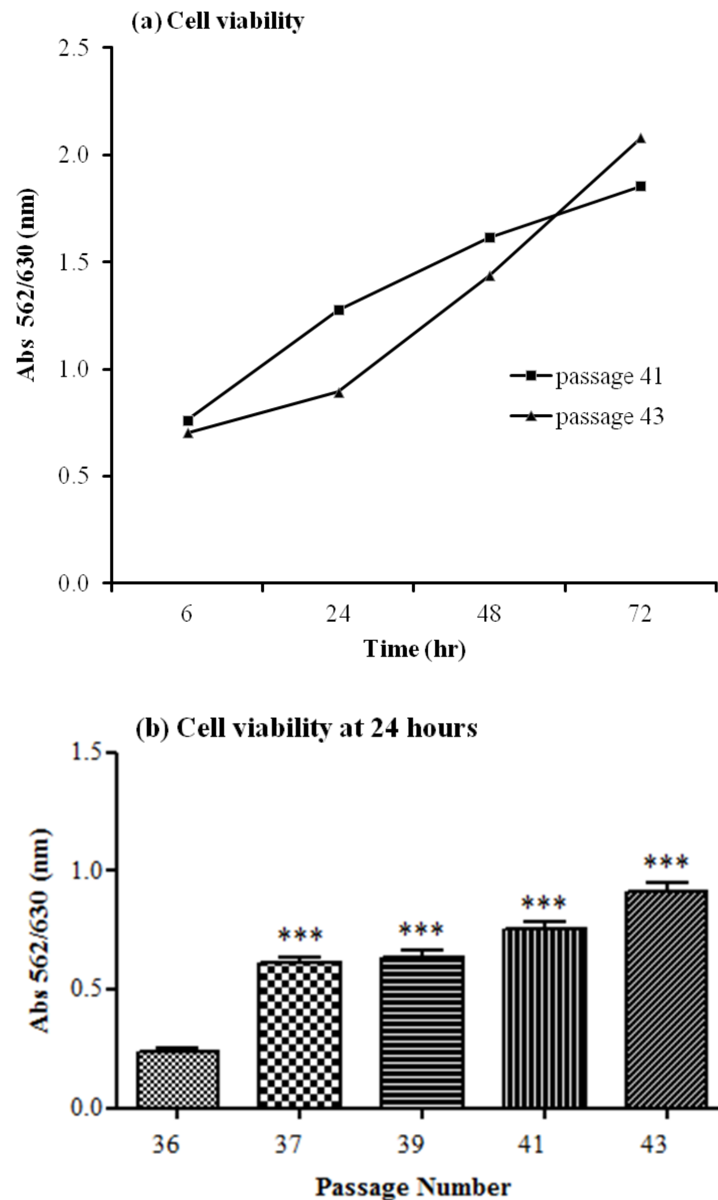


Figure 5.3 Comparison of cell viability of various cell passages. Passage 41 and 43 under control condition at 6, 24, 48 and 72 hours (a) and passage 36- 43 at 24 hours (b). HAPI cells at density $1x10^4$ cells/well on 96 well plates were cultured in DMEM supplemented with 2.5% FBS under control condition. Absorbance of formazan crystal (wavelength 562 and 630 nm) was measured at 6, 24, 48 and 72 hours. Data are presented as mean ± SEM (n=6). ***P value < 0.05 when compare to that of passage 36.

In order to evaluate the ability of HAPI cells on ROS production, the cells were activated with *tert*-buthyl hydroperoxide (t-BuOOH) and LPS.

After incubation of HAPI cells with t-BuOOH for 1 hour, cell viability was decreased and ROS production was increased in concentration dependent-manner (Figure 5.4). Increased ROS production was also observed after activation with 1 μg/ml LPS for 6 hours. The maximum ROS production was at 48 hours (Figure 5.5).

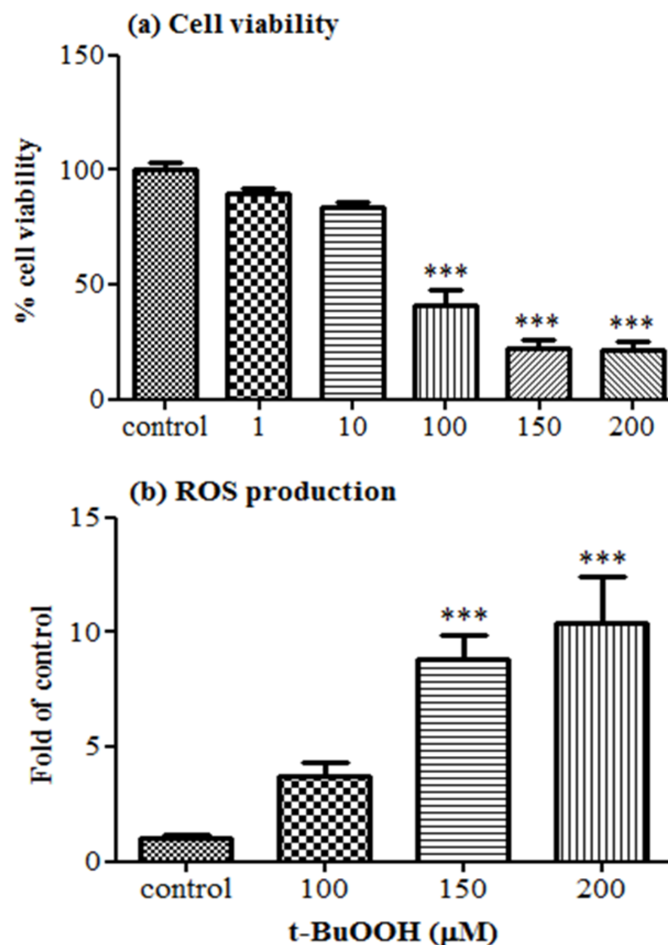


Figure 5.4 Effect of t-BuOOH on microglia viability (a) and ROS production (b). HAPI cells were incubated in medium supplement with various concentrations of t-BuOOH (1, 10, 100, 150 and 200 μM) for 1 hour before performing assay. Data are expressed as mean ± SEM at least 3 independent experiments. ***P value < 0.05 when compared with control.

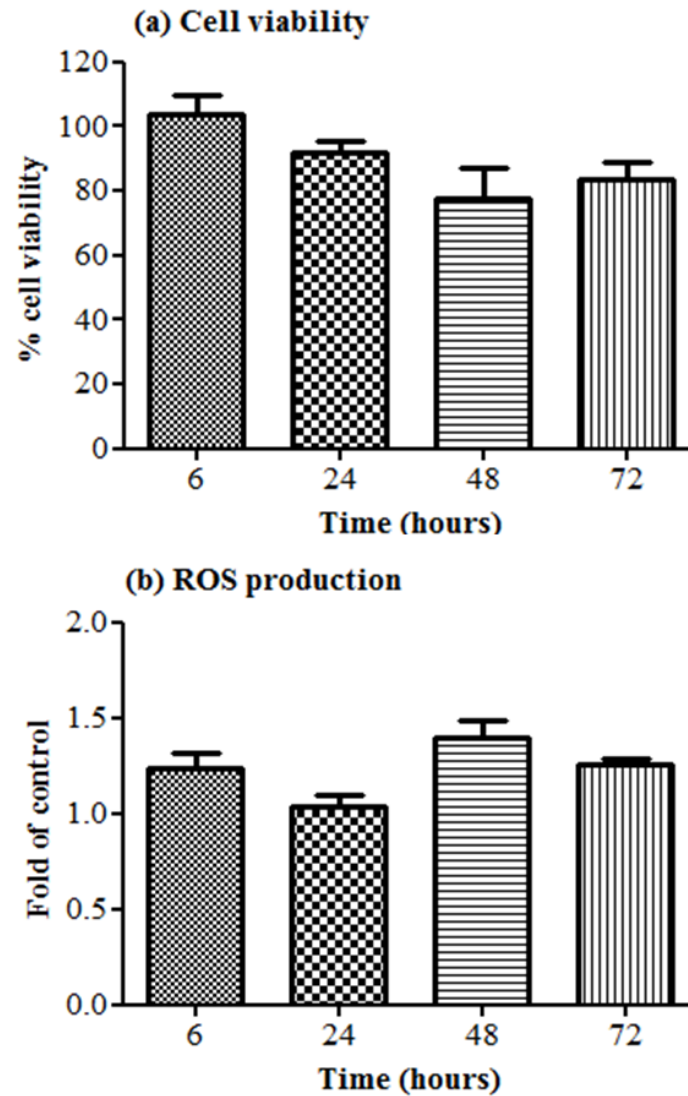


Figure 5.5 Effects of LPS on microglia viability (a) and ROS production (b).
HAPI cells were incubated in medium supplement with 1 μg/ml LPS for 6, 24, 48 and 72 hours before performing assay. Data are expressed as mean ± SEM at least 3 independent experiments.

5.2 Flavivirus-induced ROS production in HAPI cells

To study flavivirus-induced ROS production, HAPI cells were challenged with flavivirus (JEV, CxFV, DENV-2 and DENV-4) at multiplicity of infection (MOI) 0.01 and 0.1 for 2 hours absorption period. Results of cell viability and ROS production are demonstrated in Figure 5.6-5.10.

JEV decreased cell viability of HAPI cells, the significant differences were observed at 48 and 72 hrs after incubating with MOI 0.01. In addition, higher MOI (0.1) induced a significant decrease in cell viability earlier at 24 hours. JEV induced ROS production about 1.2-2 fold comparing with mock control. ROS production was observed at 6 hours and lasted up to 72 hours. ROS production at 48 and 72 hours was significant higher than that of 6 hours.

CxFV significantly decreased cell viability at 24, 48, 72 hours with MOI 0.01 and also decreased at 48 and 72 hours with MOI 0.1. CxFV induced ROS production about 1.2-1.7 fold comparing to mock control. ROS production at MOI 0.01 was as though slightly higher than MOI 0.1. ROS production at 48 hours with MOI 0.01 was showed the highest amount.

DENV-2 decreased cell viability of HAPI cells, the significant differences were observed at 48 and 72 hours with both MOI 0.01 and 0.1. ROS production induced by DENV-2 was about 1.1-1.8 fold comparing to mock control.

DENV-4 reduced viability of HAPI cells, it was significantly decreased at 48 and 72 hours after incubation with MOI 0.01. Additionally, MOI 0.1 induced progressively decreased in cell viability from 6 to 72 hours. Induction of ROS production by DENV-4 was observed about 1.2-2.3 fold comparing with mock control. ROS production was observed at 6 hours and the production was last up to 72 hours. Higher MOI (0.1) resembled to be more effective. At higher MOI, ROS production was significantly increased at 72 hours.

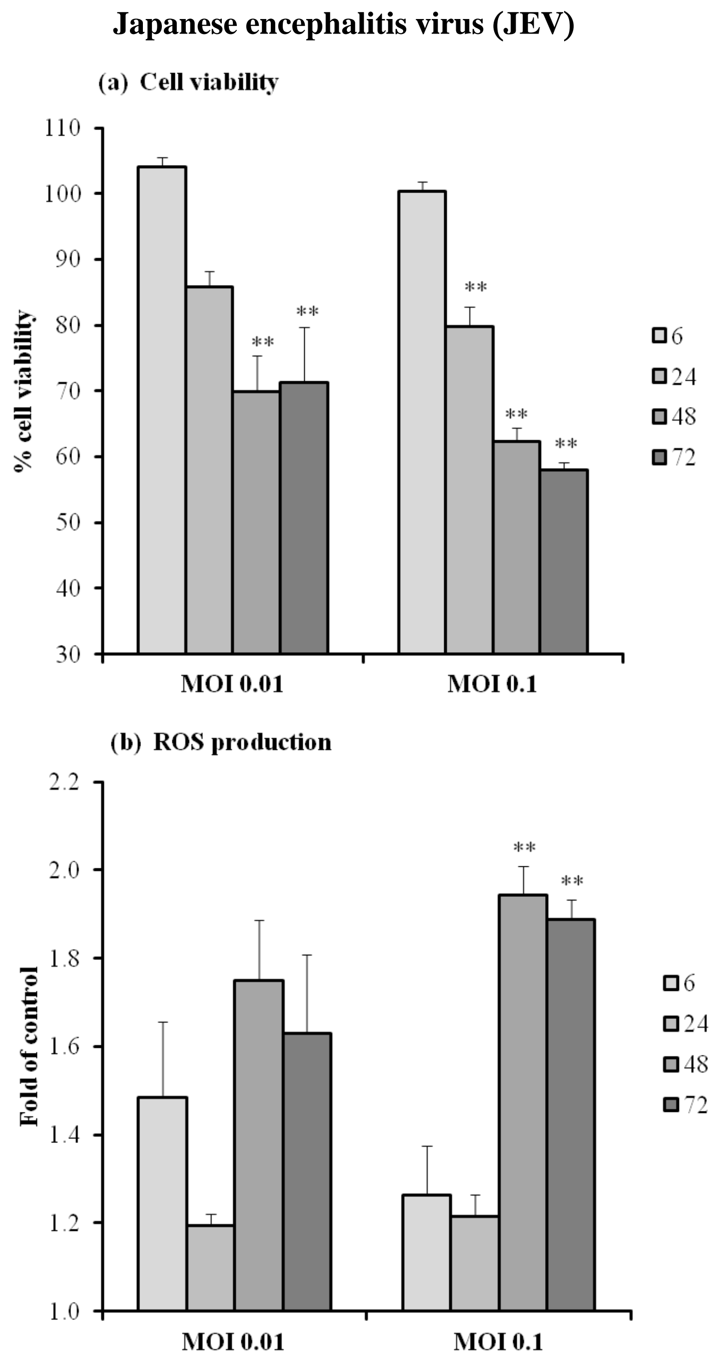


Figure 5.6 Effects of Japanese encephalitis virus (JEV) on cell viability (a) and ROS production (b) in HAPI cells. HAPI cells were cultured in medium supplemented with 2.5% FBS at density of $1x10^4$ cells/well on 96 well plate. HAPI cells were challenged with JEV at MOI 0.01 and 0.1 for 2 hours absorption period. Cell viability and ROS determination were performed at 6, 24, 48 and 72 hours. Data are expressed as mean ± SEM (n=8). *P value < 0.05, **P value < 0.001 when compared with their respective at 6 hours.

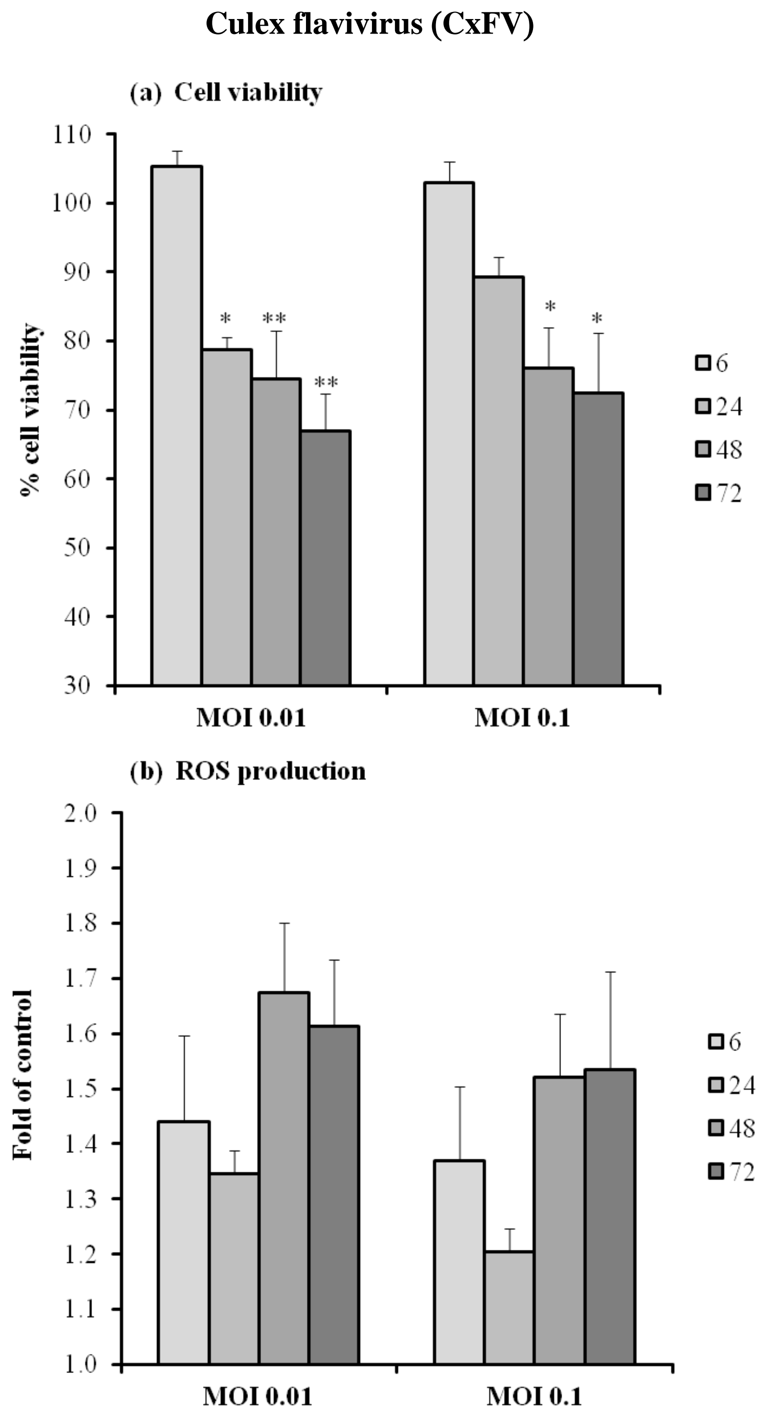


Figure 5.7 Effects of Culex flavivirus (CxFV) on cell viability (a) and ROS production (b). HAPI cells were challenged with CxFV at MOI 0.01 and 0.1 for 2 hours absorption period. Cell viability and ROS determination were performed at 6, 24, 48 and 72 hours. Data are expressed as mean ± SEM (n=8). *P value < 0.05, **P value < 0.001 when compared with their respective at 6 hours.

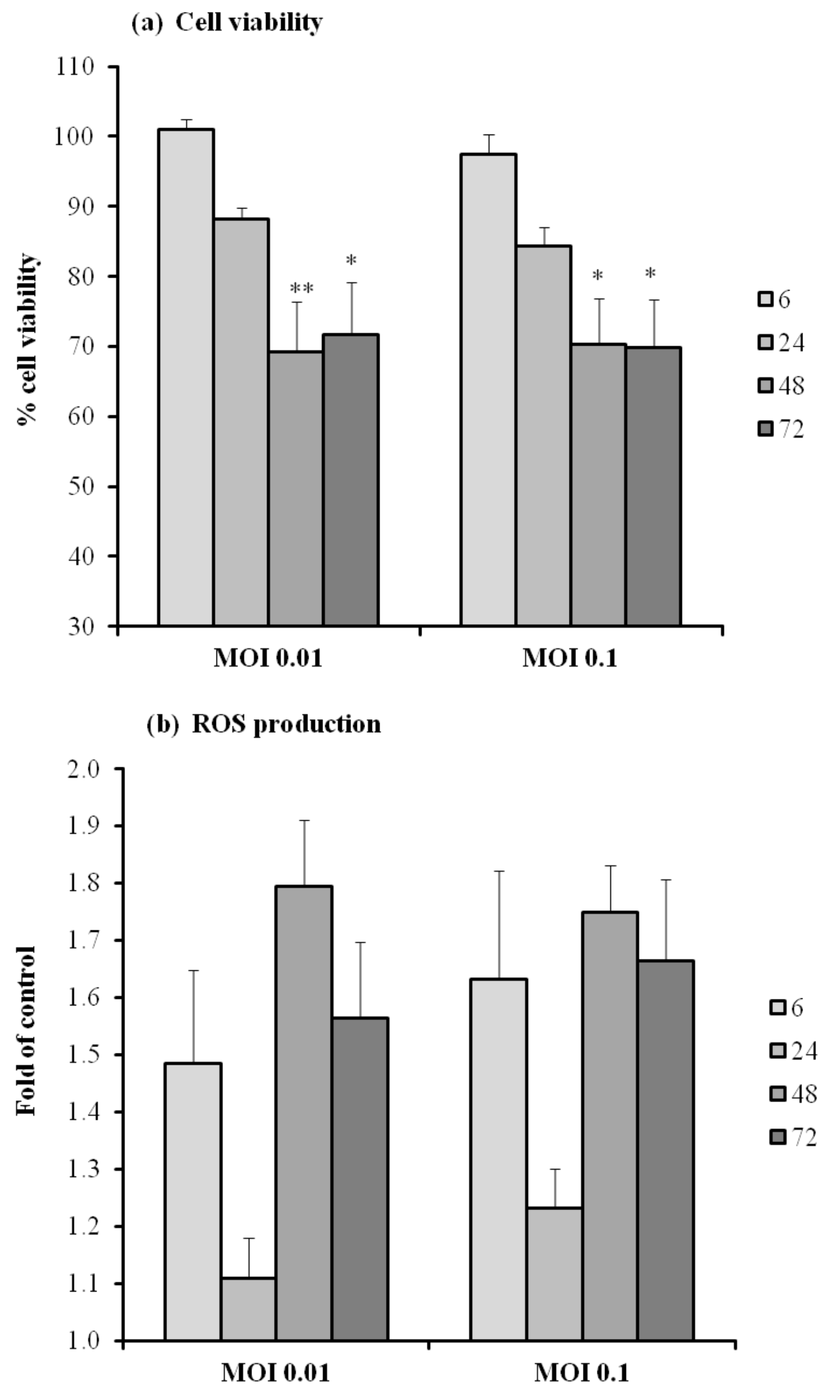


Figure 5.8 Effects of Dengue-2 virus (DENV-2) on cell viability (a) and ROS production (b). HAPI cells were challenged with DENV-2 at MOI of 0.01 and 0.1 for 2 hours absorption period. Cell viability and ROS determination were performed at 6, 24, 48 and 72 hours. Data are expressed as mean ± SEM (n=8). *P value < 0.05, **P value < 0.001 when compared with their respective at 6 hour.

Dengue-4 (DENV-4)

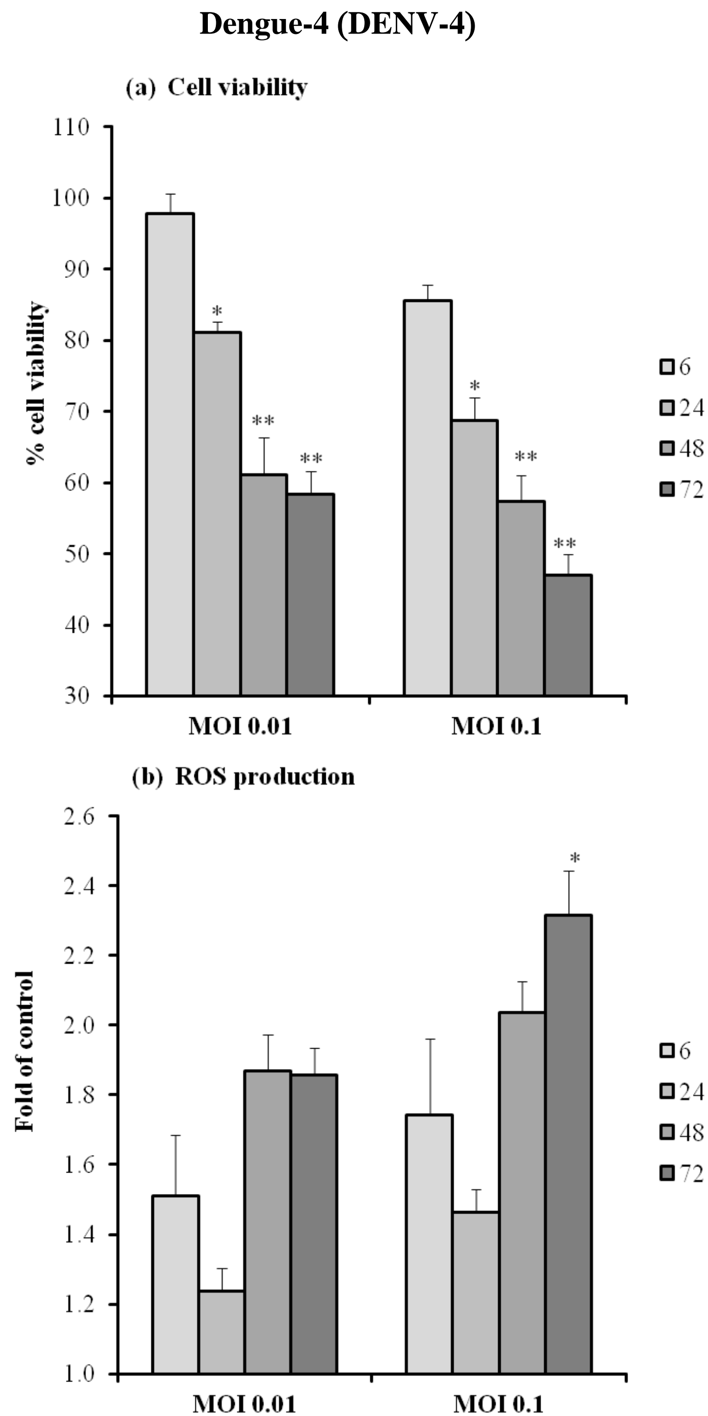


Figure 5.9 Effects of Dengue-4 virus (DENV-4) on cell viability (a) and ROS production (b). HAPI cells were challenged with DENV-2 at MOI 0.01 and 0.1 for 2 hours absorption period. Cell viability and ROS determination were performed at 6, 24, 48 and 72 hours. The data were expressed as mean ± SEM (n=8). *P value < 0.05, **P value < 0.001 when compared with their respective at 6 hour.

Figure 5.10 and 5.11 summarized time-cause effects of 4 viruses on cell viability and ROS production. All viruses decreased of cell viability in corresponding with increased ROS production. Statistical analysis (Table 5.1-5.4) demonstrated the significances of ROS production were at 48 and 72 hours. DENV-4 showed the highest efficacy on increasing ROS production, practically at 6 hours, in comparison with other viruses.

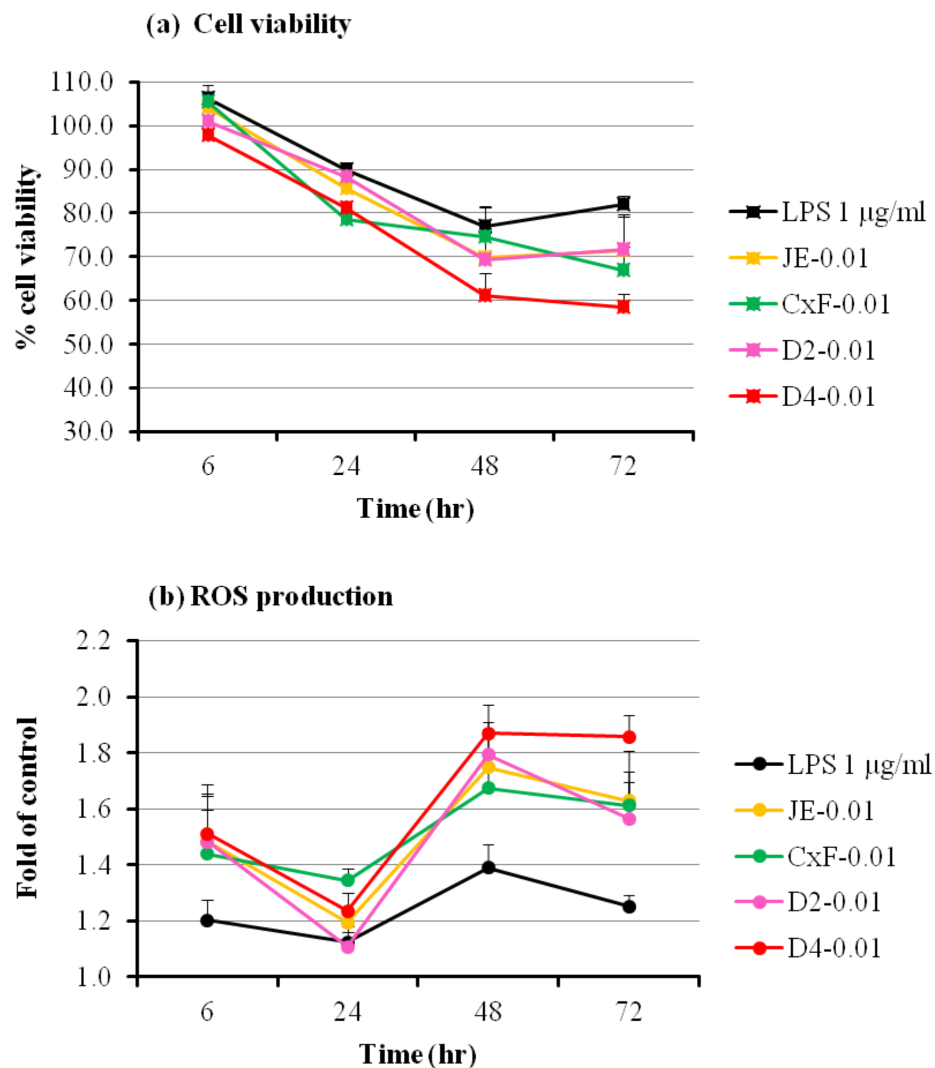


Figure 5.10 Effects of flavivirus at MOI 0.01 on microglia viability (a) and ROS production (b). HAPI cells were challenged with flavivirus at MOI of 0.01 for 2 hours absorption period. Cell viability and ROS determination were performed at 6, 24, 48 and 72 hours. Data are expressed as mean ± SEM (n=8). *P value < 0.05, **P value < 0.001 when compared with their respective at 6 hours.

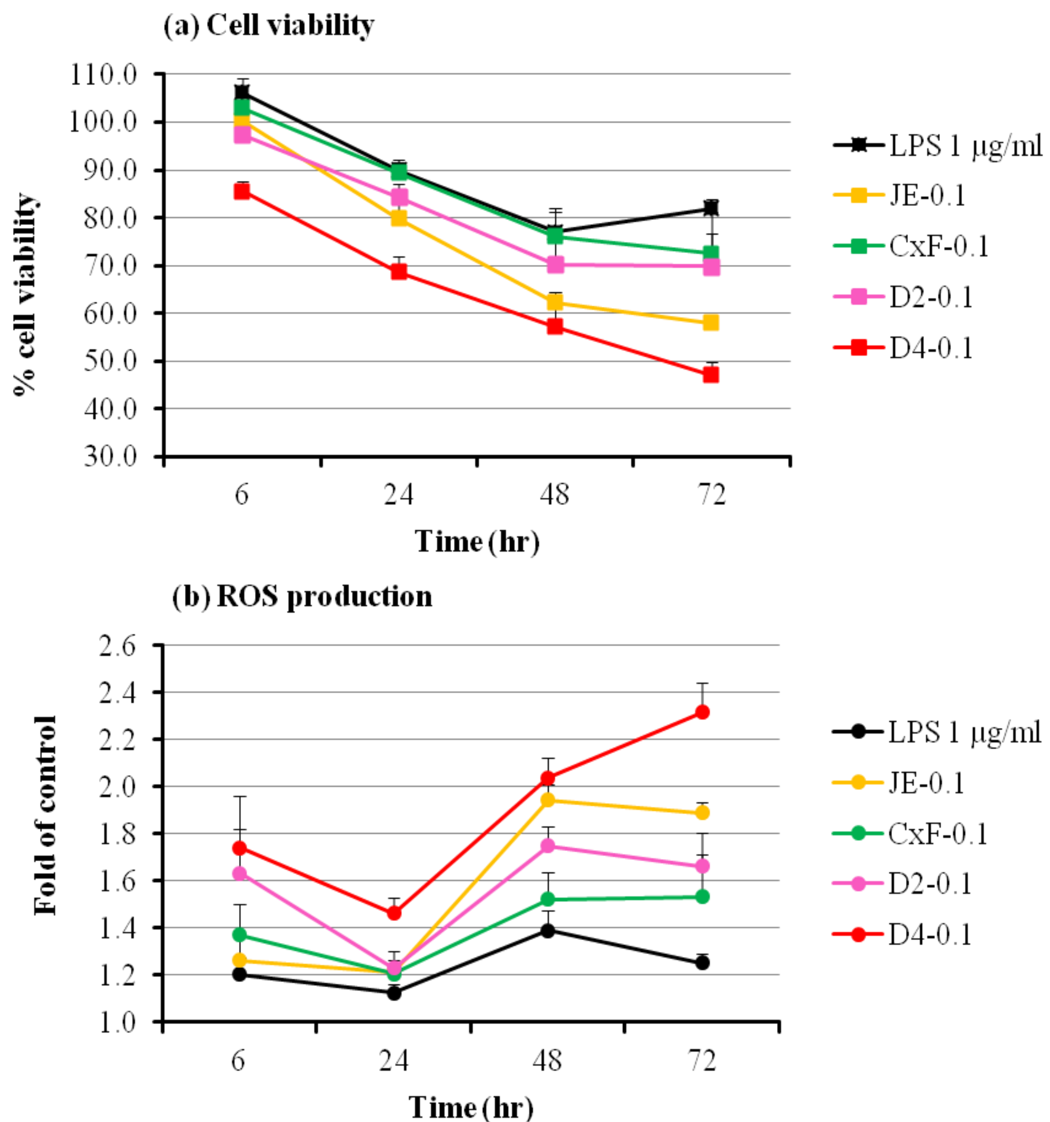


Figure 5.11 Effects of flavivirus at MOI 0.1 on microglia viability (a) and ROS production (b). HAPI cells were challenged with flavivirus at MOI of 0.1 for 2 hours absorption period. Cell viability and ROS determination were performed at 6, 24, 48 and 72 hours pi. Data are expressed as mean ± SEM (n=8). *P value < 0.05, **P value < 0.001 when compared with their respective at 6 hours.

Table 5.1: P-value of the cell viability comparisons between mock and flavivirus via ANOVA analysis following by Bonferroni as a post-test

MOCK	Time of post infection (hours)			
	6	24	48	72
JEV-0.01	ns	ns	0.000	0.001
JEV-0.1	ns	ns	0.000	0.000
CxFV-0.01	ns	ns	0.005	0.000
CxFV-0.1	ns	ns	0.011	0.006
DENV-2-0.01	ns	ns	0.000	0.001
DENV-2-0.1	ns	ns	0.000	0.000
DENV-4-0.01	ns	ns	0.000	0.000
DENV-4-0.1	0.006	0.001	0.000	0.000

ns = not significance

Table 5.2: P-value of the cell viability comparisons between DENV-4 (MOI of 0.1) and other flavivirus via ANOVA analysis following by Bonferroni as a post-test

DENV-4	Time of post infection (hours)			
	6	24	48	72
JEV-0.01	0.001	0.035	ns	ns
JEV-0.1	0.018	ns	ns	ns
CxFV-0.01	0.000	ns	ns	ns
CxFV-0.1	0.000	ns	ns	ns
DENV-2-0.01	0.009	0.001	ns	ns
DENV-2-0.1	ns	ns	ns	ns
DENV-4-0.01	ns	ns	ns	ns
mock	0.006	0.001	0.000	ns

ns = not significance

Table 5.3: P-value of the ROS production comparisons between mock and flavivirus via ANOVA analysis following by Bonferroni as a post-test

MOCK	Time of post infection (hours)			
	6	24	48	72
JEV-0.01	ns	ns	0.000	0.002
JEV-0.1	ns	ns	0.000	0.000
CxFV-0.01	ns	0.000	0.000	0.003
CxFV-0.1	ns	0.018	0.000	0.017
DENV-2-0.01	ns	ns	0.000	0.008
DENV-2-0.1	ns	0.038	0.000	0.001
DENV-4-0.01	ns	0.034	0.000	0.000
DENV-4-0.1	0.021	0.001	0.000	0.000

ns = not significance

Table 5.4: P-value of the ROS production comparisons between DENV-4 (MOI of 0.1) and other flavivirus via ANOVA analysis following by Bonferroni as a post-test

DENV-4	Time of post infection (hours)			
	6	24	48	72
JEV-0.01	ns	0.035	ns	0.004
JEV-0.1	ns	ns	ns	ns
CxFV-0.01	ns	ns	ns	0.003
CxFV-0.1	ns	ns	0.012	0.001
DENV-2-0.01	ns	0.001	ns	0.001
DENV-2-0.1	ns	ns	ns	0.009
DENV-4-0.01	ns	ns	ns	ns
mock	0.021	0.000	0.000	0.000

ns = not significance

5.3 Morphology of HAPI cells after incubation with flavivirus

Activated HAPI cells undergo shape changes from rounded-shape to ameboi shape.

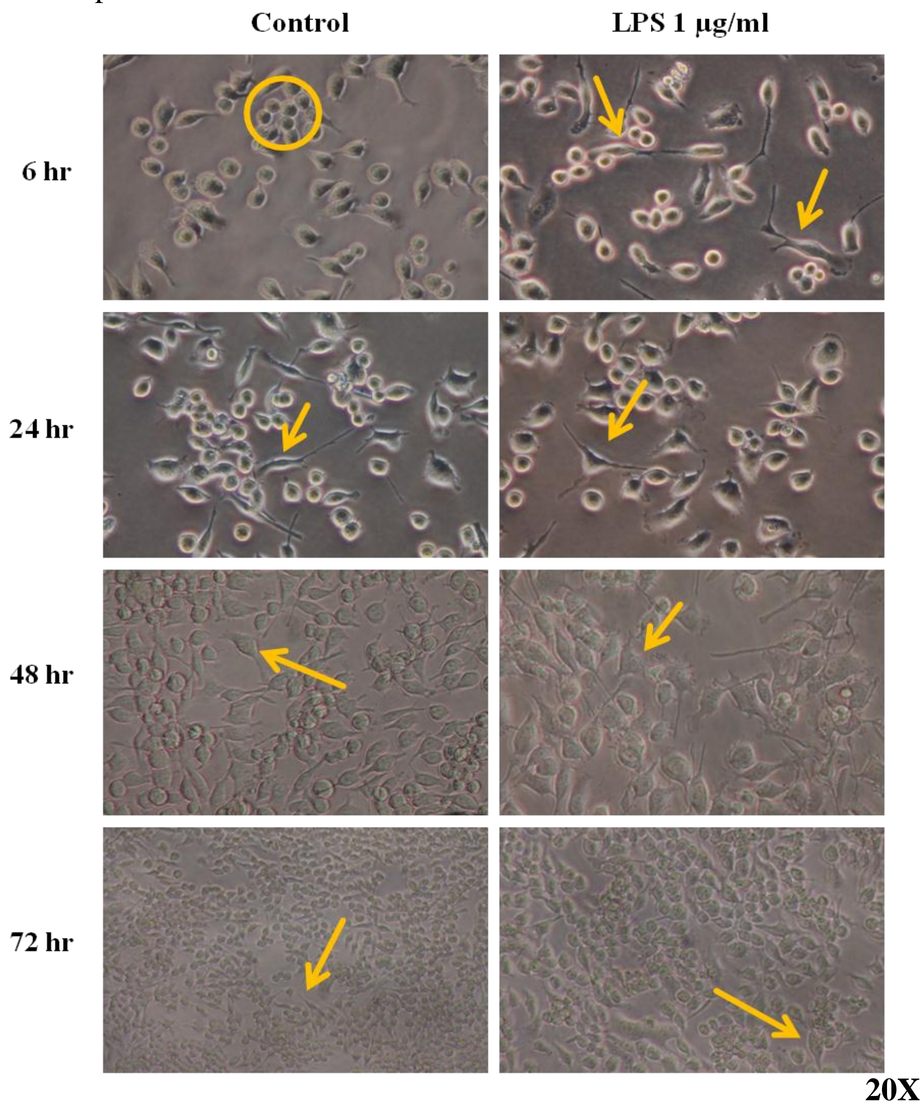


Figure 5.12 Microscopic observation of HAPI cells growth under control (untreated) and treated with LPS 1 μg/ml. HAPI cells were cultured in DMEM supplemented with 2.5% FBS at density of 1 x 10^4 cells/well on 96 well plates for 24 hours. After that, fresh medium with LPS 1 μg/ml was replaced and incubated for 2 hours. After incubation periods, medium was removed and replaced with fresh medium further incubation for 6, 24, 48 and 72 hours. Circle, shown healthy HAPI cells (rounded-shape) and arrows indicated to the activated microglia cells. The images were taken at 20X original magnification.

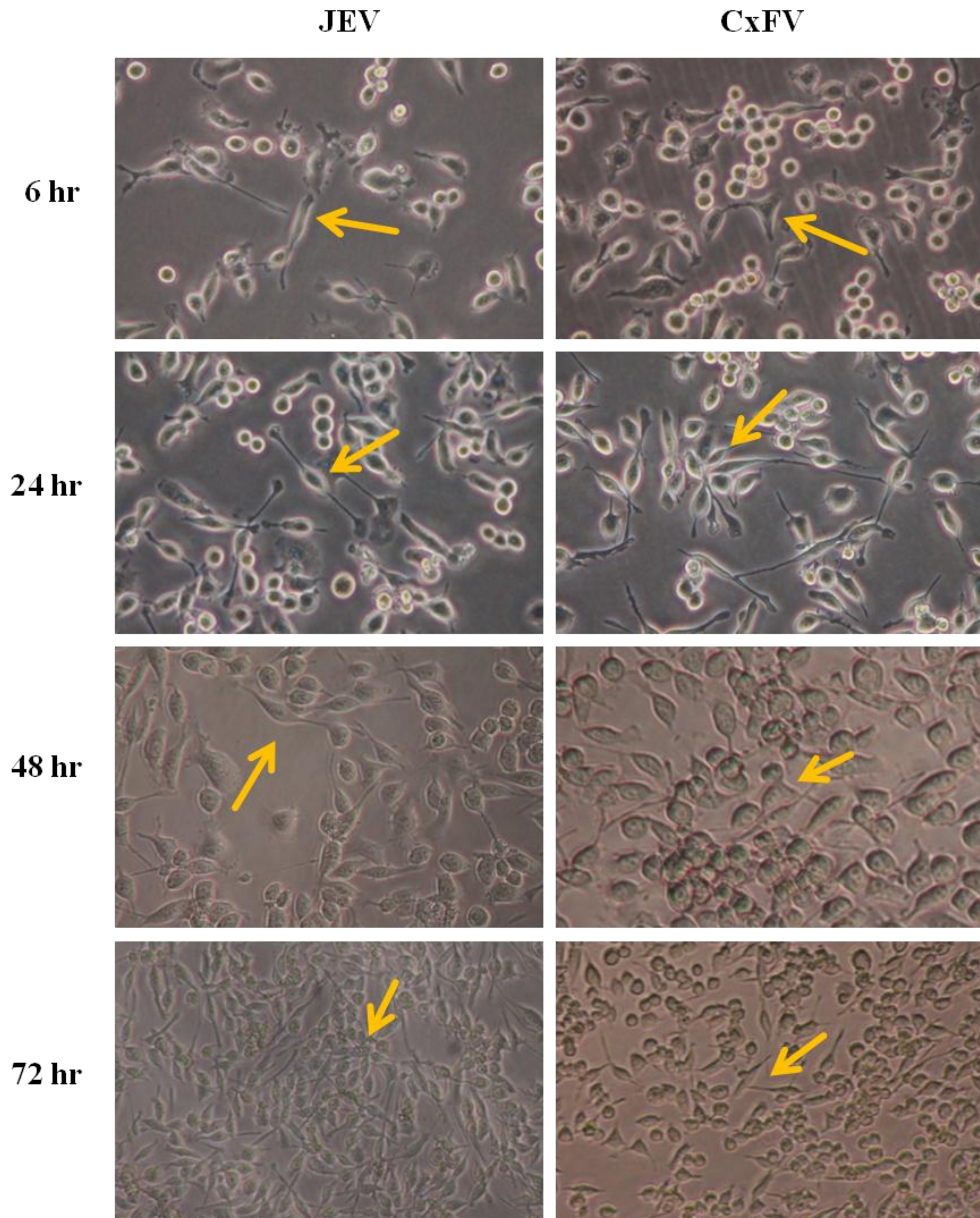


Figure 5.13 Microscopic observation of HAPI cells after activation of JEV and CxFV. HAPI cells were cultured in DMEM supplemented with 2.5% FBS at density of 1 x 10^4 cells/well on 96 well plate for 24 hours before challenging with JEV and CxFV at MOI 0.1 for 2 hours absorption period. The images were taken at 20X original magnification at 6, 24, 48 and 72 hours. Arrows indicate activated HAPI cells.

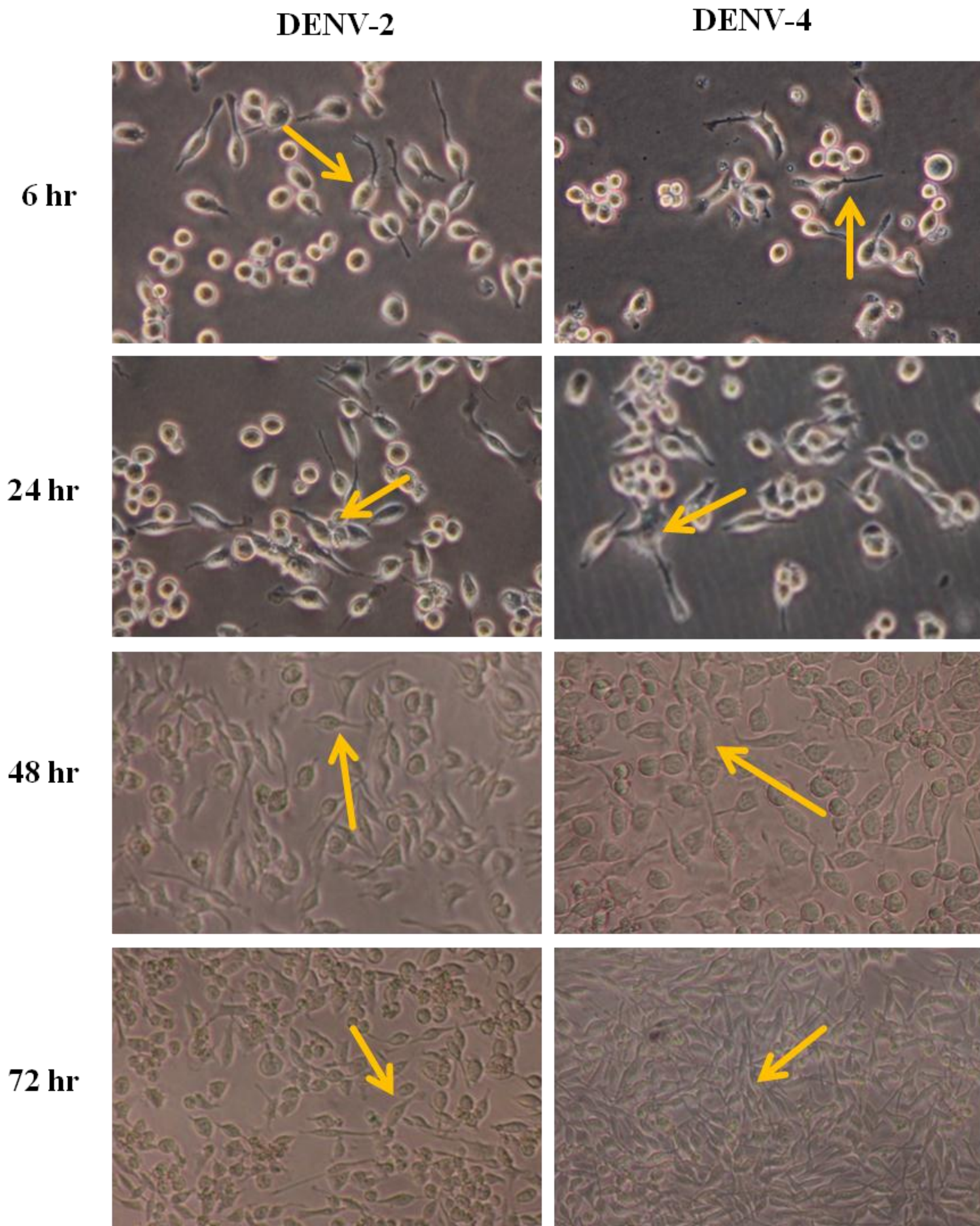


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Figure 5.14 Microscopic observation of HAPI cells after activation of DENV-2 and DENV-4. HAPI cells were cultured in DMEM supplemented with 2.5% FBS at density of 1 $x10^4$ cells/well on 96 well plate for 24 hours before challenging with DENV-2 and DENV-4 at MOI 0.1 for 2 hours absorption period. The images were taken at 20X original magnification at 6, 24, 48 and 72 hours. Arrows indicate activated HAPI cells.

5.4 Response of HAPI cells followed flavivirus infection

5.4.1 Effects of flavivirus infection on iNOS expression in microglia cells.

Activation of HAPI cells induced iNOS expression. This experiment, 1 μg/ml of LPS was used as positive control. At 24 hours after incubation with LPS, iNOS expression was increased by 1.4 fold. As well as LPS, iNOS expression was significantly increased (1.4 fold increase) after activation with JEV, CxFV and DENV-2. On the other hand, DENV-4 did not induce iNOS expression.

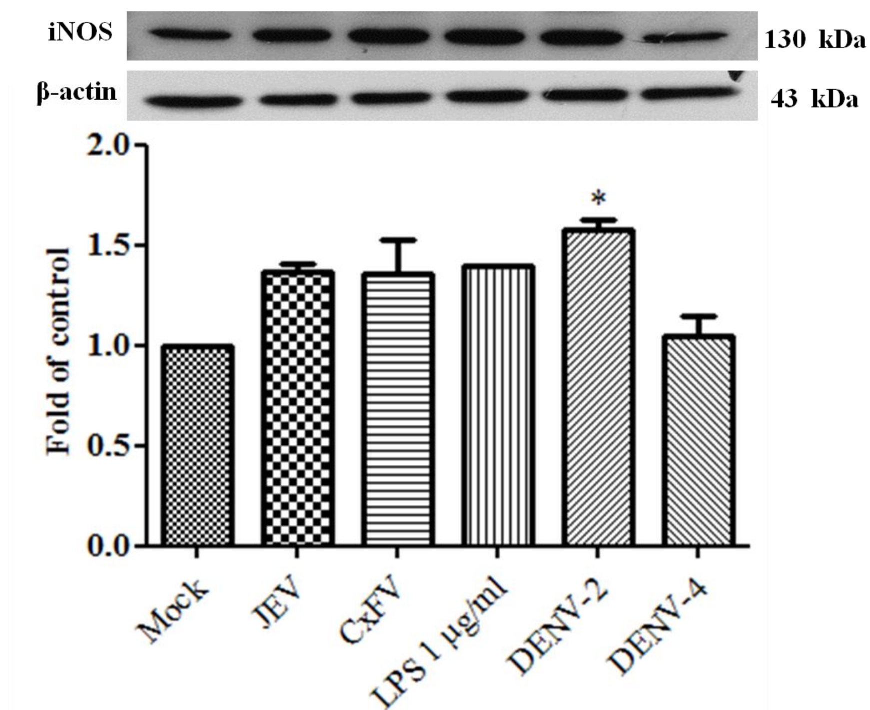

Figure 5.15 Effect of flavivirus activation on iNOS expression in HAPI cells. Expression of iNOS was analyzed at 24 hours after activation with flavivirus (MOI 0.1). β-actin was used as internal control. Data are expressed as means ± SEM (n=3). *P < 0.05 when compared with control.

5.4.2 Effects of flavivirus on caspase-3 expression and activation in microglia cells.

Caspases are proteases essential for apoptosis and inflammation. Effects of flavivirus on apoptotic pathways were examined. Western blot analysis demonstrated the slightly increased of caspase-3 expression after JEV and CxFV activation. Activation of caspase-3 yield an active form of caspase-3 (cleaved caspase-3, 17 kDa). Cleaved caspase-3 was trifling increased in JEV, CxFV and DENV-2, but comparable with LPS activation. On the other hand, active form of caspase-3 was suppressed in DENV-4.

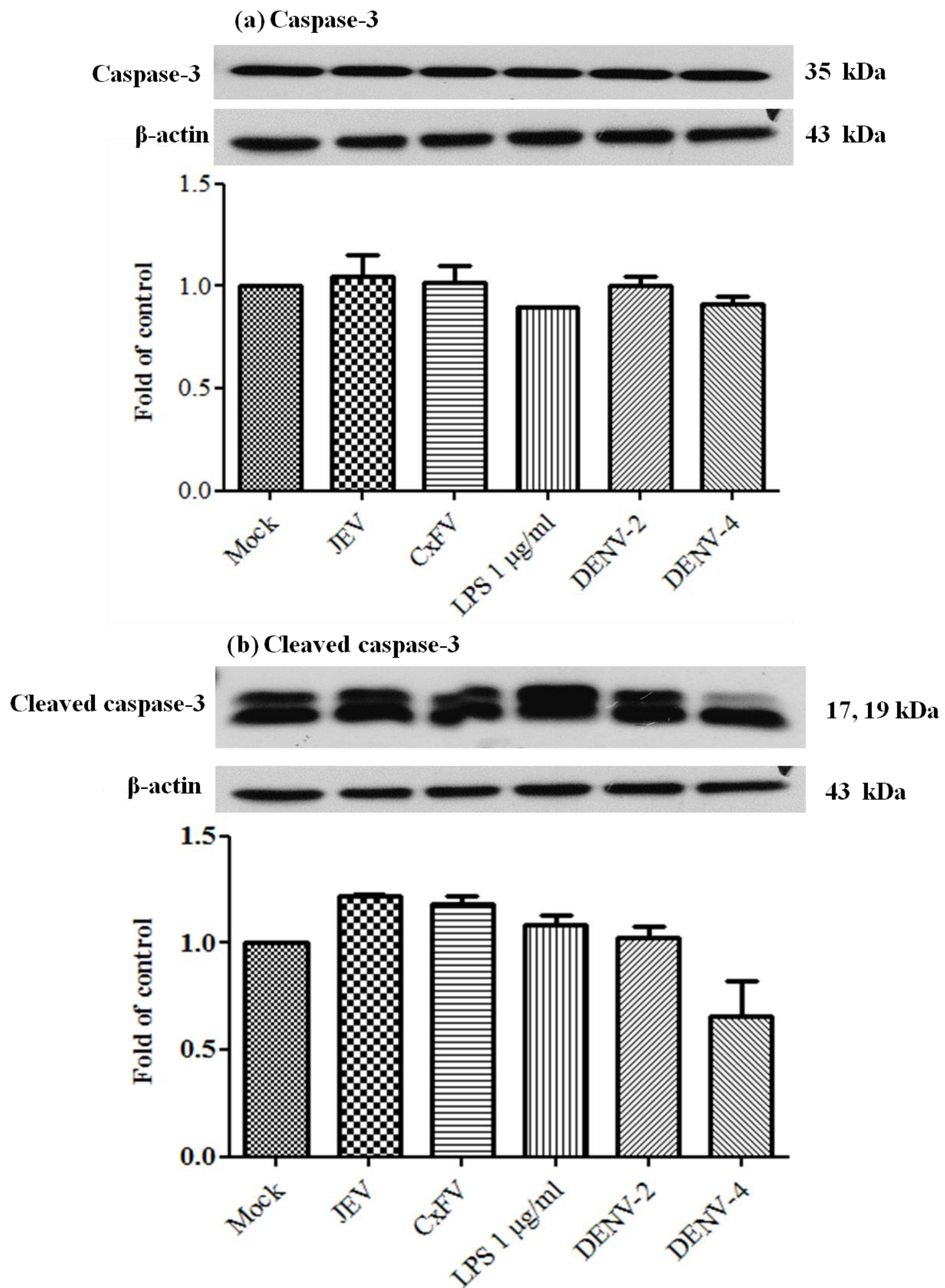

Figure 5.16 Effects of flavivirus on caspase-3 expression (a) and activation of cleaved caspase-3 (b) in HAPI cells. Caspase-3 expression and cleaved caspase-3 was analyzed at 24 hours after activation with flavivirus (MOI 0.1). β-actin was used as internal control. Data are expressed as means ± SEM (n=3).

5.4.3 Release of interleukin-1β (IL-1β)

Normally, interleukin-1β is pro-inflammatory cytokines that is secreted in low level in activated microglia cells. IL-1β was measured in very low level after HAPI cells activated with flavivirus. Induction of secretion by flavivirus at MOI 0.1 revealed that at 6 hours IL-1β level was around 100 -125 pg/ml and at 24 hours was 63-82 pg/ml (Table 5.5).

Table 5.5: Release of IL-1β

	IL-1β (pg/ml)	
Flavivirus	6 hours pi	24 hours pi
JEV	123.76 ± 10.03	76.86 ± 7.95
CxFV	120.90 ± 8.25	81.86 ± 3.98
DENV-2	112.10 ± 4.19	70.90 ± 10.93
DENV-4	100.19 ± 6.94	79.00 ± 13.80