CHAPTER IV
RESULTS

4.1 Propagation of JEV and virus titration

The Japanese encephalitis virus (JEV) was propagated in C6/36 cells at an MOI of 1. After process of viral absorption was taken for 2 hr at 28°C with constant shaking, cells were supplemented with fresh culture medium and incubated at 28°C for 5 days. Aliquots of the culture medium were stored at -80°C. The JEV titer was later determined by standard plaque assay on LLC-MK2 cells. Cells were seeded in 6 well plates and cultured under standard condition for 2 days before time. The serial dilution of virus suspension was directly infected onto LLC-MK2 monolayer for 2 hr at 37°C with constant shaking. Later on, Seakem LE agarose mixed with nutrient overlay was added to each well. The plates were further incubated at 37°C in 5% CO_2 atmosphere. At day 7, cells were fixed with 3.7% paraformaldehyde for 1 hr at room temperature. Agarose were removed and plaques were visualized by 1% crystal violet in ethanol staining. The virus titer was calculated as 1.10×10^7 p.f.u./ml (Figure 4.1).

4.2 Growth curve analysis of JEV-infected CHME-5 cells

To study the growth dynamics of CHME-5 cells in response to JEV infection, CHME-5 cells were mock-infected or JEV-infected at an MOI of 10 and 100. Cells were allowed to grow for 5 days. The attached cells were trypsinized and pooled together with the detached cells before counting as a total cell number at 24 hr interval for 5 consecutive days in order to observe the effect of JEV infection to the cell growth rate. Live and dead cell numbers were determined by trypan blue dye exclusion assay. Infection with JEV at an MOI of 10 produced a relatively small deficit in total cell number, compared to that of mock-infected CHME-5 cells, from day 3 post infection (Figure 4.2).

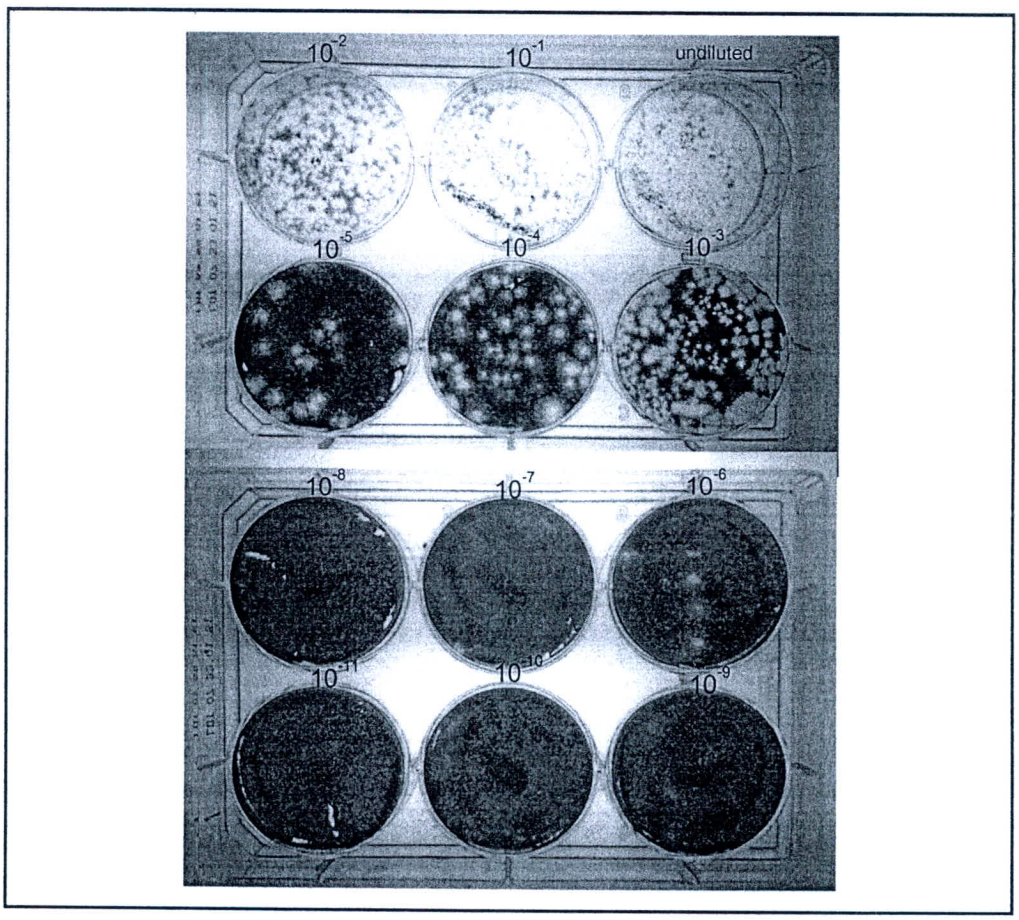

Figure 4.1 Standard plaque assay of JEV on LLC-MK-2

This figure shows virus titration on LLC-MK-2 cells. Cells were seeded in 6 –well plates ($4X10^5$ cells /well) before being infected with 12-fold serially diluted JEV viral suspension and subsequently overlaid with 2X nutrient agar. The plates were incubated for 7 days. The cells were fixed with 3.7% formaldehyde and agar was removed. Plaques were counted, after staining with 1% crystal violet in ethanol, for virus titer calculation. Each well represented a dilution of virus stock as indicated.

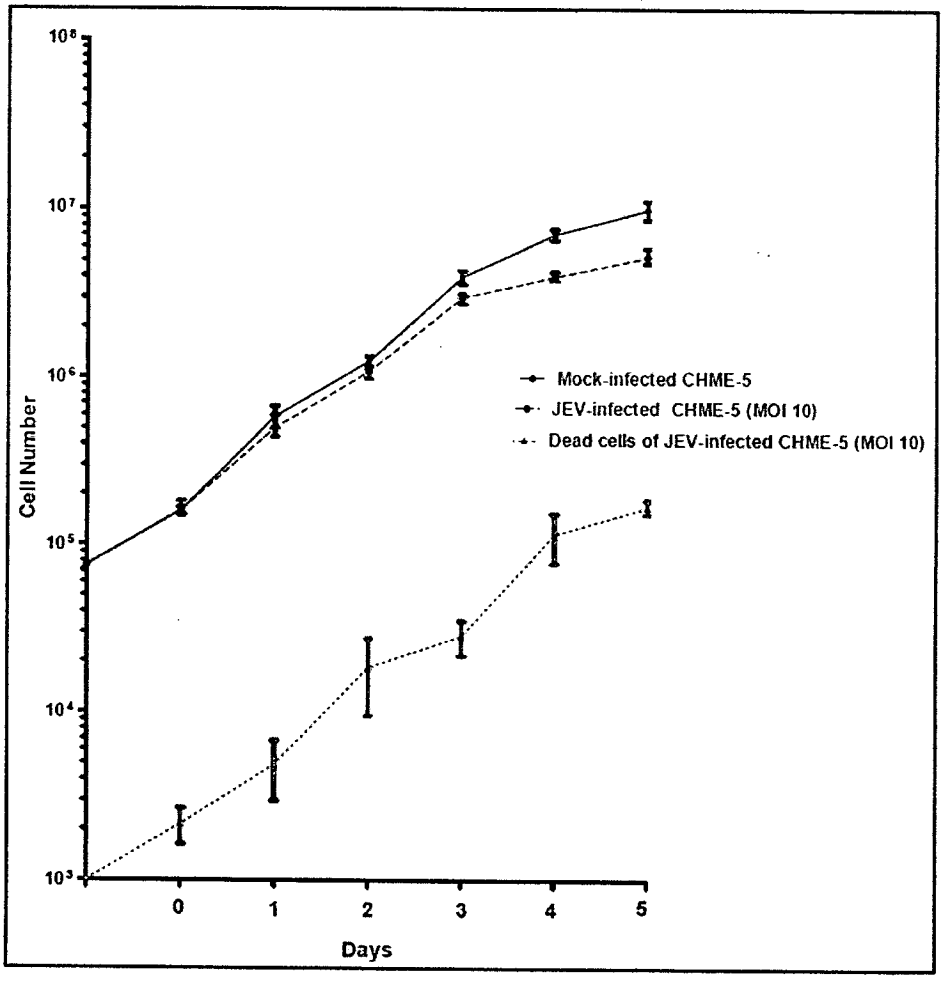

Figure 4.2 Growth curves of mock-infected and JEV-infected CHME-5 cells (MOI of 10)

This figure shows the growth curves of mock-infected CHME-5 cells compared to that of JEV-infected CHME-5 cells. $7.5x10^4$ CHME-5 cells were seeded onto 6 well- plates before time. The cells were infected with JEV at an MOI of 10 or mock-infected at day 0. Total cell number in each condition was determined up to 5 days post infection. The solid line and thin dashed line represented the total number of mock-infected cells and JEV-infected cells with an MOI of 10 respectively. The number of dead cells identified by tryptan blue staining was also separately plotted into the graph (thin dotted line). The values represent the mean ± SEM of three independent experiments.

However, three-fold difference in total cell number of JEV-infected CHME-5 cultures with an MOI of 100, compared to that of mock-infected CHME-5 cells was detected since day 2 post infection (Figure 4.3). Surprisingly, the percentage of cell death comparing between JEV –infected CHME-5 cultures with different MOI was relatively the same during the time of the experiment (less than 10%). These results indicate a slower proliferation rate in JEV-infected microglial culture with a higher multiplicity of infection. The data correlated well with the morphological changes, observed under light microscope (Figure 4.4). Mock-infected CHME-5 cultures displayed rod-shaped morphology with a few number of detached cells (panel b). In contrast, JEV-infected CHME-5 cells were transformed to activated states with amoeboid-shaped appearance (panel h).

4.3 Determination of the percentage of infectivity

The susceptibility of human microglia to JEV infection was directly visualized by indirect immunofluorescence. CHME-5 cells were either mock-infected or infected with JEV at an MOI of 100 at 37 ^{o}C for 2 hr and incubated under standard condition for 48 hr. The virus antigen was clearly detected in cytoplasm of JEV- infected microglia using a pan specific anti-Flavivirus monoclonal antibody raised against E protein and FITC –conjugated goat anti-mouse IgG antibody as a primary and secondary antibody respectively (Figure 4.5). In order to examine the involvement of autophagy in response to JEV infection in human microglial cells, the infectious condition need to be firstly optimized in order to obtained, at least, an estimated of 70% infectivity. CHEM-5 cells were either mock-infected or infected with JEV at an appropriate MOI of infection at 37 ^{o}C for 2 hr and incubated under standard condition for 12, 24 and 48 hr. The percentage of viral infectivity was determined using immunocytochemistry. Positive staining cells were identified as dark brown cells under light microscope using a pan specific anti-Flavivirus monoclonal antibody raised against E protein and HRP–conjugated goat anti-mouse IgG antibody as a primary and secondary antibodies respectively (Figure 4.6). The number of positive cells was randomly counted (20 fields per slide). No immunoreactive cell was observed in mock-infected CHME-5 sample (panel a-c). From the results (Figures 4.6 and 4.7), the increasing number of positive staining cells were detected during the time course of infection, in both infectious condition of CHME-5 cells were infected with JEV respectively at day 2 post infection. The result indicated the optimal condition that is suitable for autophagy study (an MOI of 100, at day 2 post infection).

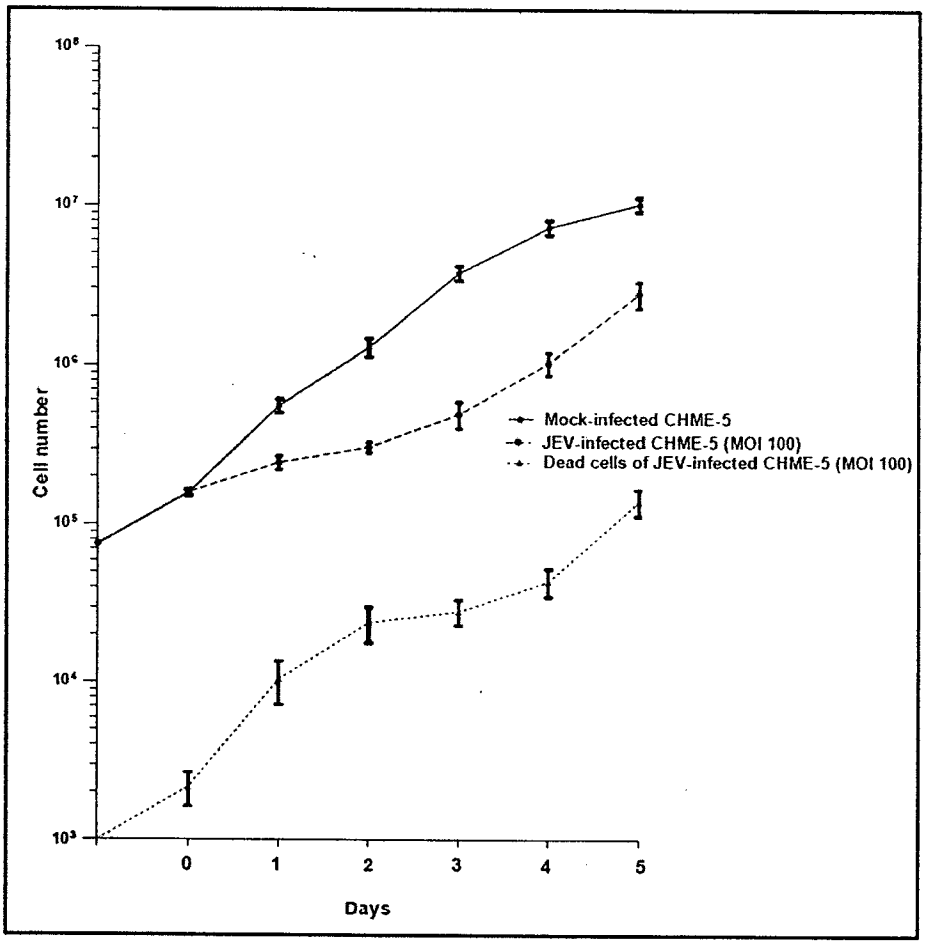

Figure 4.3 Growth curves of mock-infected and JEV-infected CHME-5cells (MOI of 100)

This figure shows the growth curves of mock-infected CHME-5 cells compared to that of JEV-infected CHME-5 cells. $7.5x10^4$ CHME-5 cells were seeded onto 6 well- plates before time. The cells were infected with JEV at an MOI of 100 or mock-infected at day 0. Total cell number in each condition was determined up to 5 days post infection. The solid line and thin dashed line represented the total number of mock-infected cells and JEV-infected cells with an MOI of 100 respectively. The number of dead cells identified by tryptan blue staining was also separately plotted into the graph (thin dotted line). The values represent the mean ± SEM of three independent experiments.

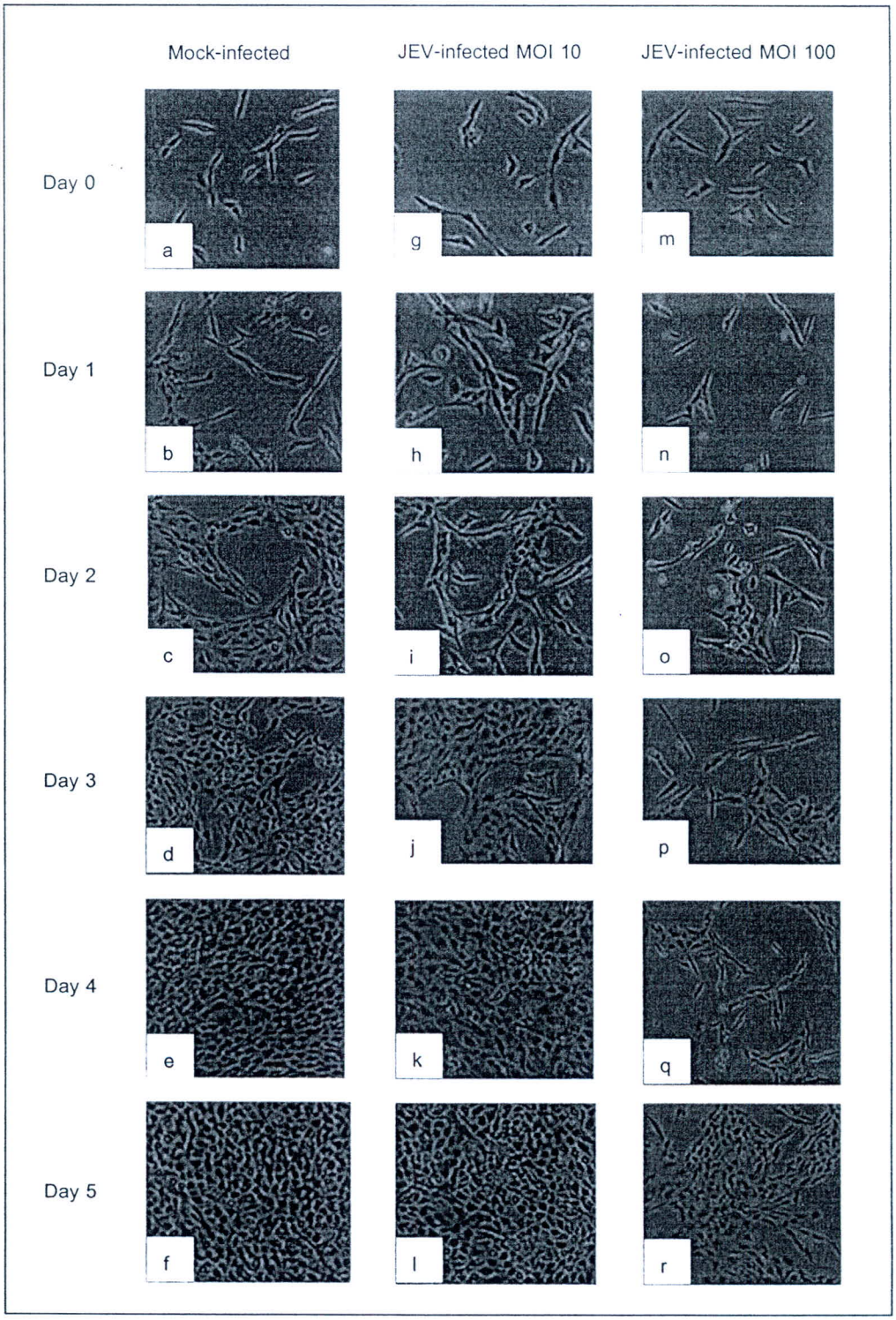
Mock-infected
JEV-infected MOI 10
JEV-infected MOI 100
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Figure 4.4 Morphological alterations of JEV-infected CHME-5 cells

This figure represents morphological changes of JEV-infected CHME-5 cells compared with mock-infected CHME-5 cells. $7.5x10^4$ CHME-5 cells were seeded onto 6 well-plates. Cells were infected with JEV at an appropriate MOI as indicated or mock-infected. Cells were further incubated at 37°C with 5% CO_2 atmosphere for different intervals and were observed under a light microscope (X100). Cells were mock-infected (a-f), infected with JEV at an MOI of 10 (g-l) or infected with JEV at an MOI of 100 (m-r).

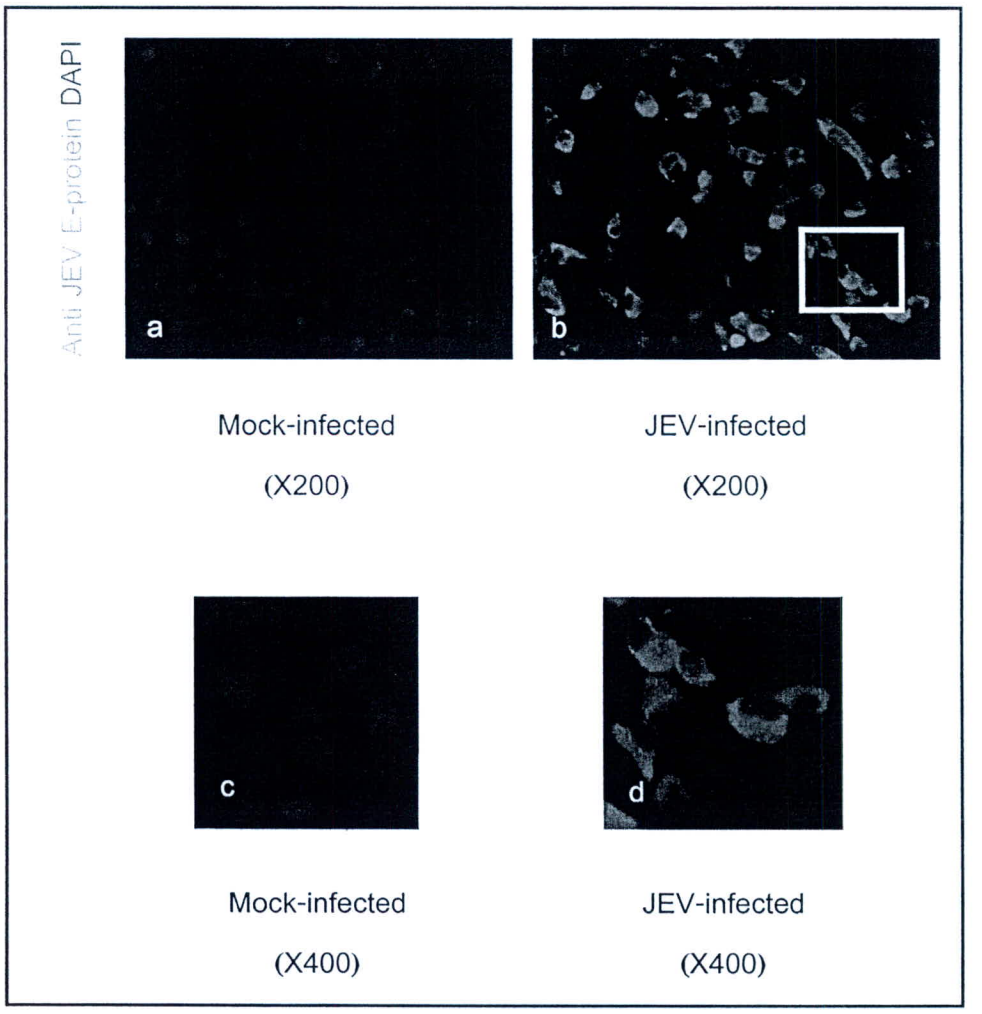

Figure 4.5 JEV infection of CHME-5 cells.

This figure shows the indirect immunofluorescence staining of JEV-infected CHME-5 cells. CHME-5 cells were either mock-infected (a,c) or infected with JEV at an MOI of 100 (b,d) and incubated for 48 hr. Cells were subsequently incubated with a pan specific anti-flavivirus monoclonal antibody raised against E protein followed by a FITC-conjugated goat anti-mouse IgG secondary antibody (green). Nuclei were counterstained with DAPI (blue). The coverslips were then mounted onto glass slides using Prolong Gold antifad e.. The fluorescent signals were observed under fluorescence microscope.

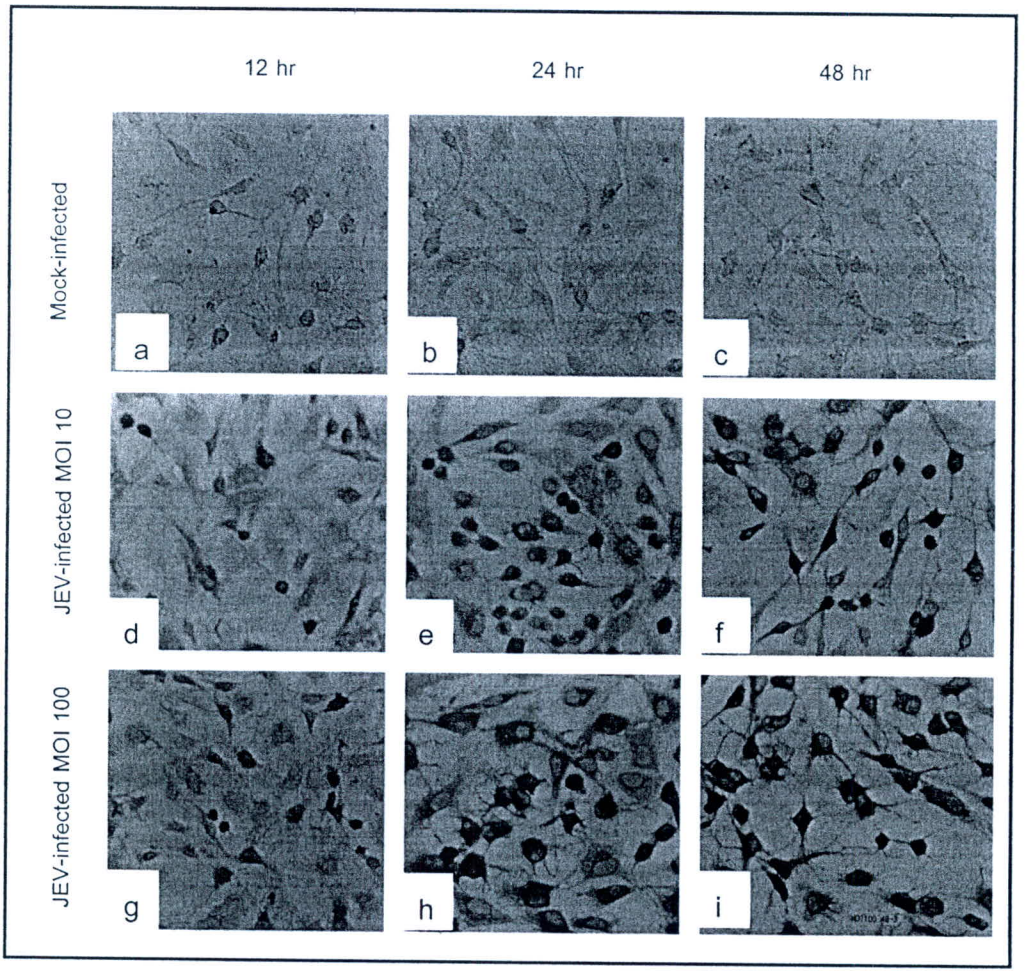

Figure 4.6 Immunocytochemistry staining of JEV-infected CHME-5 cells.

This figure illustrates the immunocytochemistry staining of JEV-infected CHME-5 cells. CHME-5 cells were either mock-infected (a-c) or infected with JEV at an MOI of 10 (d-f) or MOI of 100 (g-i) and incubated further for 12, 24 and 48 hr as indicated. Cells were subsequently incubated with a pan specific anti-flavivirus monoclonal antibody raised against E protein followed by a HRP-conjugated goat anti-mouse IgG secondary antibody. Samples examined under a light microscope. Magnification X400

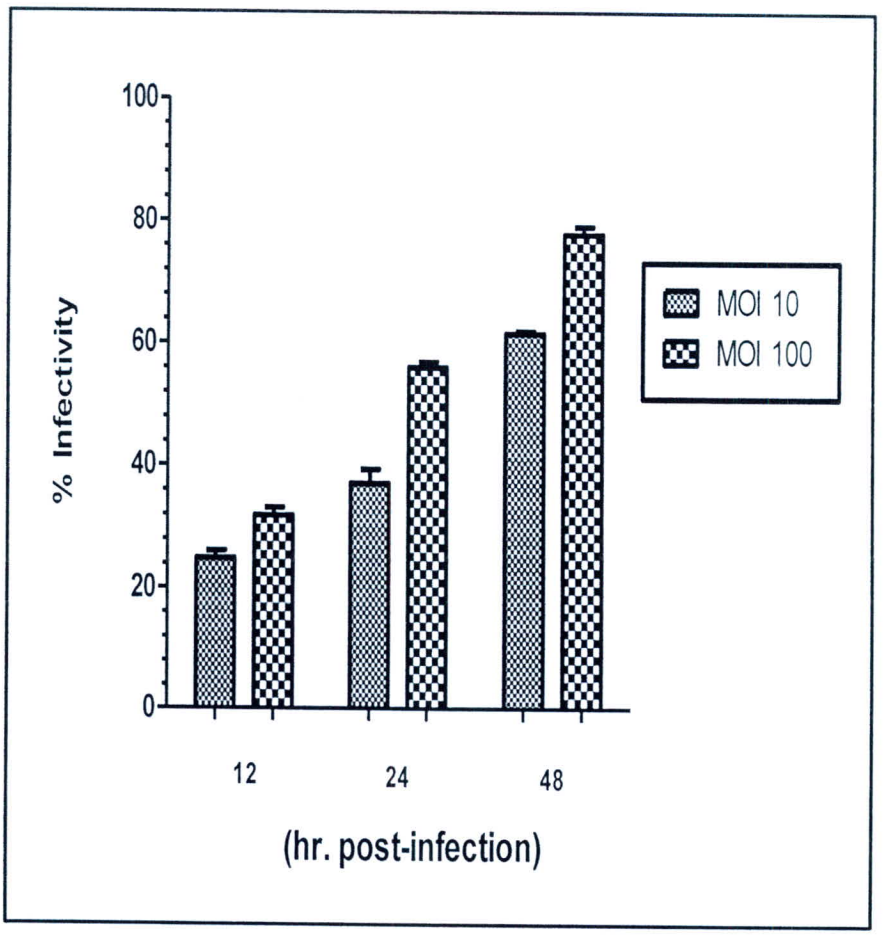

Figure 4.7 Histogram statistics of the percent infectivity determined by immunocytochemistry in JEV-infected CHME-5 cells

This figure shows histogram statistics of the percent infectivity determined by immunocytochemistry in JEV-infected CHME-5 cells as illustrated in Figure 4.6.The number of positive cells was counted (20 fields per slide) and plotted as a bar graph. The values represent the mean ± SEM of two independent experiments.

4.4 Autophagy in JEV-infected human microglial cells

4.4.1 Detection of autophagy by transmission electron microscope

To determine whether JEV infection triggers an autophagy in human microglial cells or not, ultrastructure analysis of JEV-infected CHME-5 cells using transmission electron microscope was performed. Ultrastructural study of mock-infected CHME-5 cells demonstrated cells with normal appearance of the nuclei and cell membrane. Nucleus appeared as a rounded or ovoid shape with a thin rim condensed chromatin. In cytoplasm, several cytoplasmic organelles including rER, ribosome and mitochondria were observed (Figure 4.8).

In contrast, electron microscopic examination of JEV-infected cells demonstrated cells with several ultrastructural alterations. Some infected cells displayed nuclei with clumping of chromatin. The cytoplasm were filled with deteriorated organelles including swollen mitochondria and dilated rER. The presence of multiple cytoplasmic membrane-bound vesicles containing portions of undigested organelles or virion-like particles were also observed (Figure 4.9).

Interestingly, several electron dense of JEV virions with a diameter of 50 nm were also observed in some autophagosomes under examination with higher magnification (Figure 4.10).

4.4.2 Detection of autophagy by indirect immunofluorescence staining

To further investigate autophagy induction in response to JEV infection in CHME-5 cells, Cells were either mock-infected or infected with JEV at an MOI of 100. At day 2 post infection, cells were processed for indirect immunofluorescence of LC3. Generally, newly synthesized LC3 is immediately cleaved and is presented in the cytosolic as LC3-I, which is diffusely distributed throughout the cytoplasm. Upon autophagy induction, some LC3-I is converted into LC3-II which integrated into autophagic membranes. This can be observed as a punctuate staining, representing autophagosome, in the cytoplasm. From the results, a significantly increment in the percentage of punctuate positive cells was observed in JEV – infected CHME-5 cells, compared to that of mock-infected cells (Figure 4.11). Double staining –immunofluorescence was performed by using antibodies against LC3 and JEV E protein. The E protein positive staining indicated JEV-infected cells. Comparing to mock-infected CHME-5 cultures, an increase in LC3 expression was also observed, co-localized in cytosol with E protein, in JEV-infected CHME-5 cells (Figure 4.12).

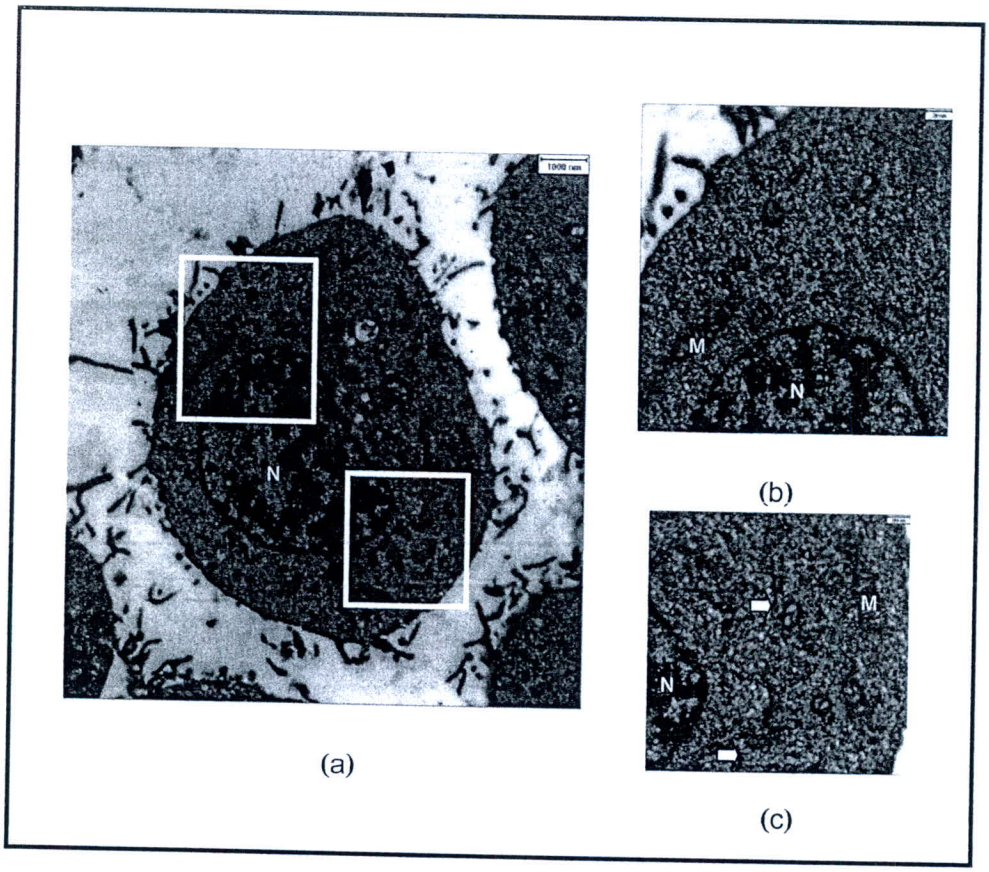

Figure 4.8 Electron micrographs of mock-infected CHME-5 cells

This figure represents electron micrographs of mock-infected CHME-5 cells. Electron microscopic examination in mock-infected CHME-5 cell demonstrates the normal appearance of cell with an oval nucleus (a). At higher magnification, the normal appearance of several cellular organelles are demonstrated (b-c). N: nucleus, M: mitochondria, White arrows: rER

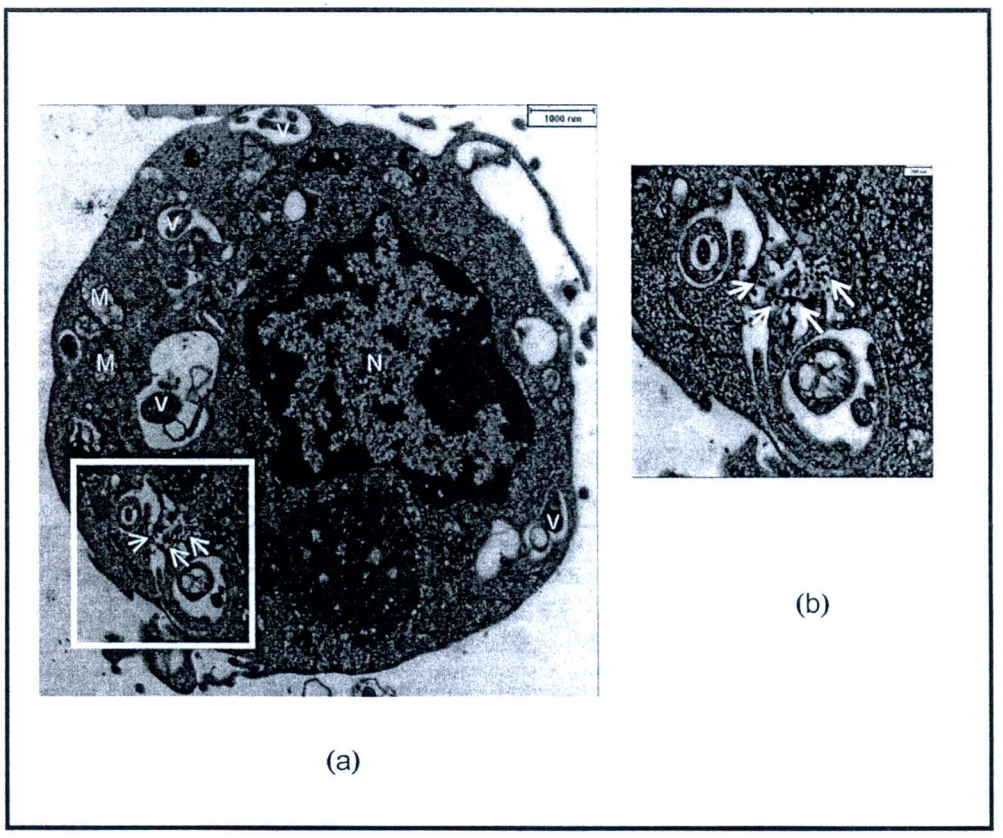

Figure 4.9 Electron micrographs of JEV-infected CHME-5 cells

This figure represents electron micrographs of JEV-infected CHME-5 cells. At day 2 post infection, JEV-infected CHME-5 cells (MOI of 100) were collected and processed for ultrastructural examination. The presence of many vesicles of a variable sizes that enclose degraded organelles are observed in JEV-infected CHME-5 cell (a). From enlargement, numerous JEV viral particles (white arrows) are demonstrated in cytoplasm (b). N: nucleus, M: mitochondria, V: vesicle

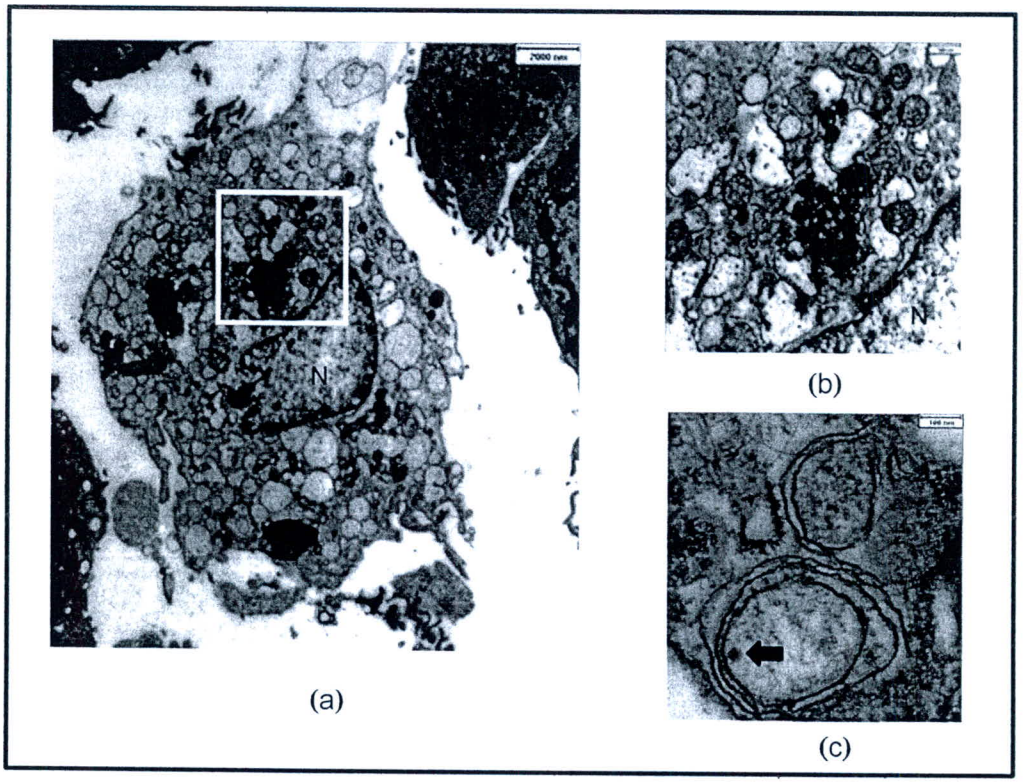

Figure 4.10 Autophagasomes in JEV-infected CHME-5 cells

This figure represents electron micrographs of JEV-infected CHME-5 cells . At day 2 post infection, JEV-infected CHME-5 cells (MOI of 100) were collected and processed for ultrastructural examination. (a) Electron micrograph of JEV-infected cell structures is significantly different from mock-infected cells. (b) At higher magnification, the infected cells present a large number of two closely apposed membrane or double-membranous vesicles with clear content reminiscent of autophagosomes. (c) One autophagosome containing a JEV virion (black arrow) with a diameter of 50 nm is observed after enlargement. N: nucleus

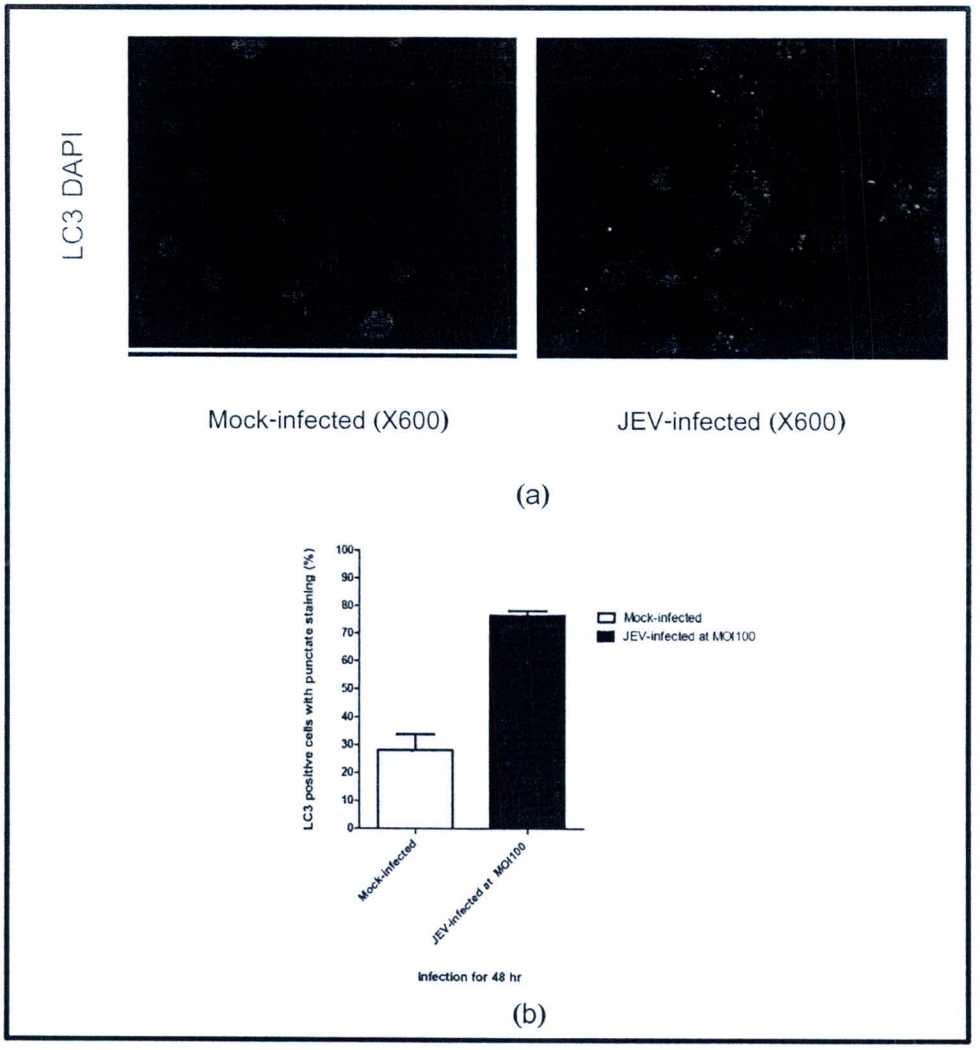

Figure 4.11 Induction of autophagy in JEV-infected CHME-5 cells

This figure represents an induction of autophagy upon JEV infection in CHME-5 cells using indirect immunofluorescence. (a) CHME-5 cells were grown on cover slips for 24 hr. Cells were later either mock-infected or infected with JEV at an MOI of 100 and incubated for 48 hr. Cells were subsequently incubated with primary antibody against LC3 followed by a Swine anti-rabbit IgG-TRITC. Nuclei were counterstained with DAPI (blue). Samples were examined under a fluorescence microscope. Magnification X600. (b) The number of LC3 positive cells were counted (20 fields per slide) and plotted as the percent positive cells with a punctuate staining. Error bars represent SEM of three independent replicates.

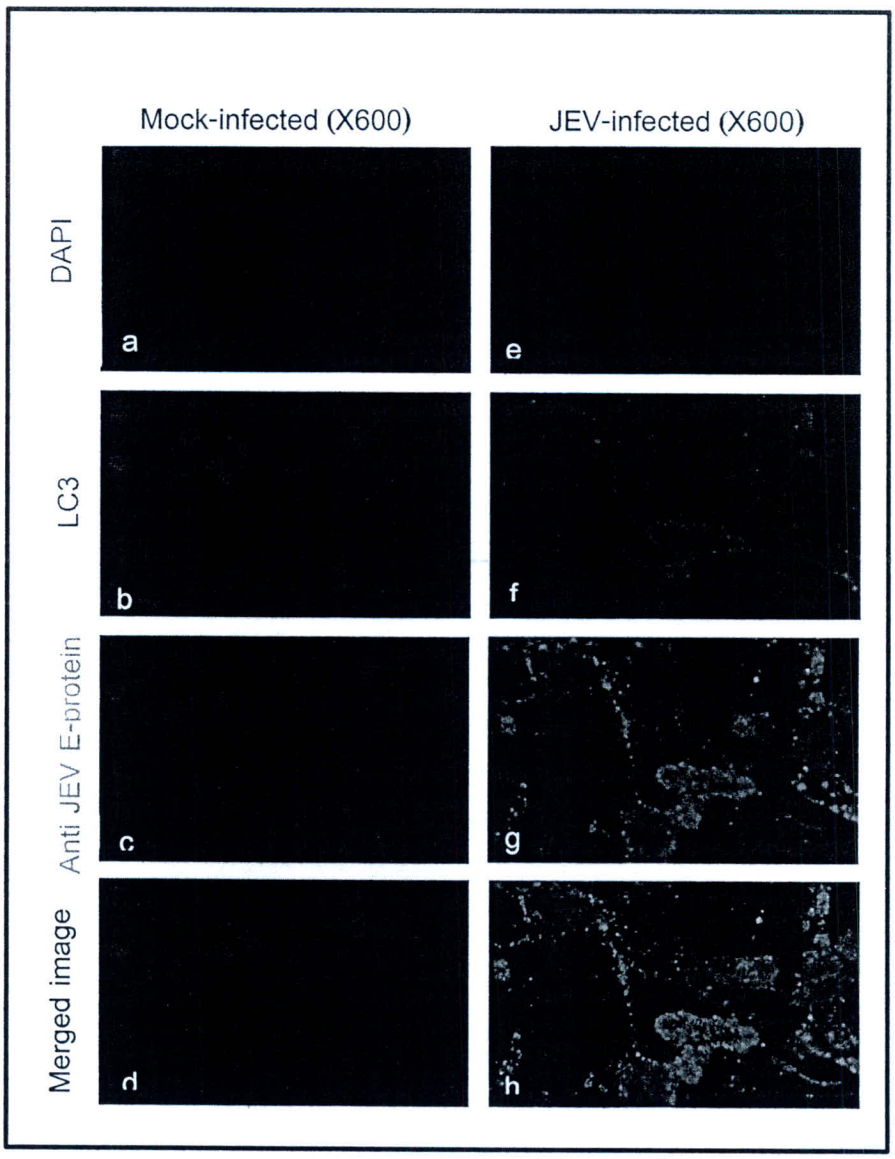

Figure 4.12 Co-localization of LC3 and JEV E-protein in JEV-infected CHME-5 cells

This figure represents the co-localization of LC3 and JEV E protein in JEV-infected CHME-5 cells using indirect immunofluorescence. CHME-5 cells were grown on cover slips for 24 hr. Cells were either mock-infected or infected with JEV at an MOI of 100 and incubated for 48 hr. Cells were subsequently double stained using antibodies against LC3 and JEV E protein. Nuclei were counterstained with DAPI. Fluorescent signals were examined under fluorescence microscope. Images were merged for DAPI (Blue), LC3 (Red) and JEV E protein (green) to examine co-localization in mocked-infected (a-d) and JEV-infected CHME-5 cells (e-h). Magnification X600

4.4.3 Detection of autophagy by Western blotting

To confirm an induction of autophagy upon JEV infection in CHME-5 cells, Western blot analysis of LC3 was performed. The cytosolic LC3-I is cleaved and conjugated with phosphatidylethanolamine to form a membrane-bound LC3, (LC3-II) upon autophagy induction. The ratio of LC3-II to actin is a direct indicator of the accumulation of the autophagosomes. An increase of LC3-II was observed in response to JEV infection. Furthermore, the human microglial cells infected with JEV at an MOI of 10 showed less induction of LC3-II than that was observed in JEV-infected cells with an MOI of 100 (Figure 4.13 and Figure 4.14).

4.5 Effect of autophagy modulation to JEV production

To characterize the role of autophagy in CHME-5 cells upon JEV infection, 3-methyladenine (3-MA), an inhibitor of type III phosphatidyl inositol 3 kinase, was used to inhibit the autophagic sequestration. Approximately $2x10^6$ CHME-5 cells were grown in 75-cm^2 tissue culture flasks for 24 hr. Cells were infected with JEV at an MOI of 100 in the condition with or without 10 mM 3-MA pretreatment. The supernatants were collected to determine the extracellular virion production. The results showed a significant reduction of virion production in response to 3-MA pretreatment (Figure 4.15). The data suggested that autophagy promoted virus replication in JEV-infected CHME-5 cells.

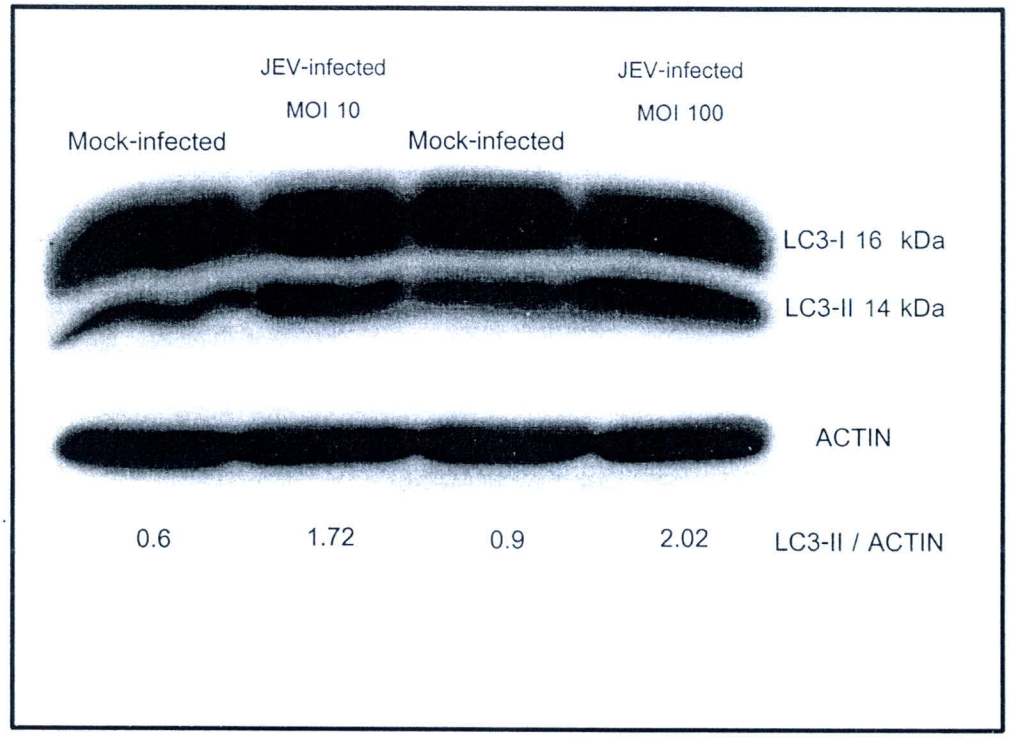

Figure 4.13 Western blotting of LC3 expression in CHME-5 cells

This figure represents western blotting of CHME-5 cell lysate separated on 15% SDS-PAGE before transferred onto a PVDF membrane. CHME-5 cells were mock-infected or infected at MOI 10 and 100. Cells were harvested at 2 days post infection for protein extraction. Western blot analysis of LC3 was performed. Expression level of Actin was shown as an internal control. Band intensity was analyzed using Quantity One software. The ratio of LC3-II / Actin intensity volume is indicated.

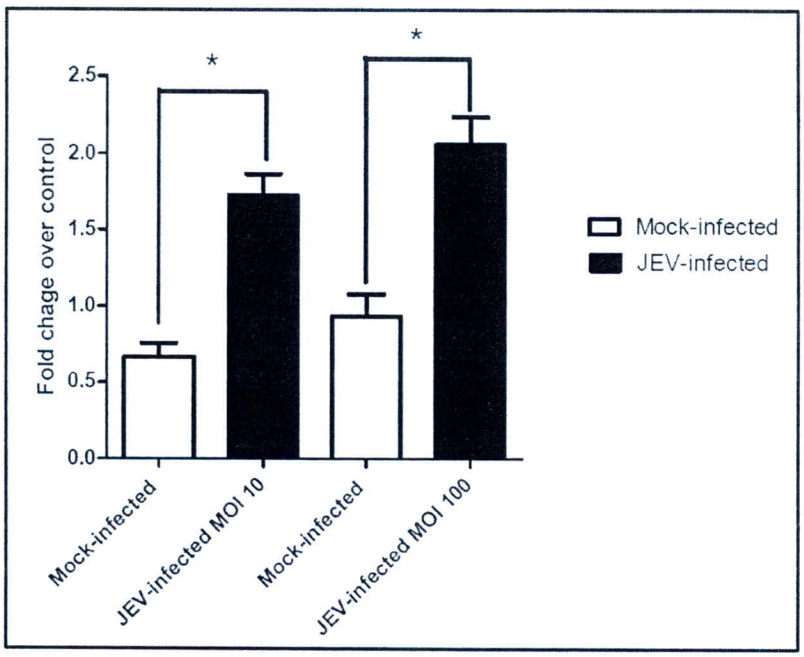

Figure 4.14 Histogram statistics of western blotting analysis of LC3-II / Actin expression in CHME-5 cells

This figure represents histogram statistics of western blotting of LC3-II / actin expression as illustrated in Figure 4.13. The statistics analysis is shown as a bar graph. The values represent the mean ± SEM from three independent experiments (*, $p < 0.05$)

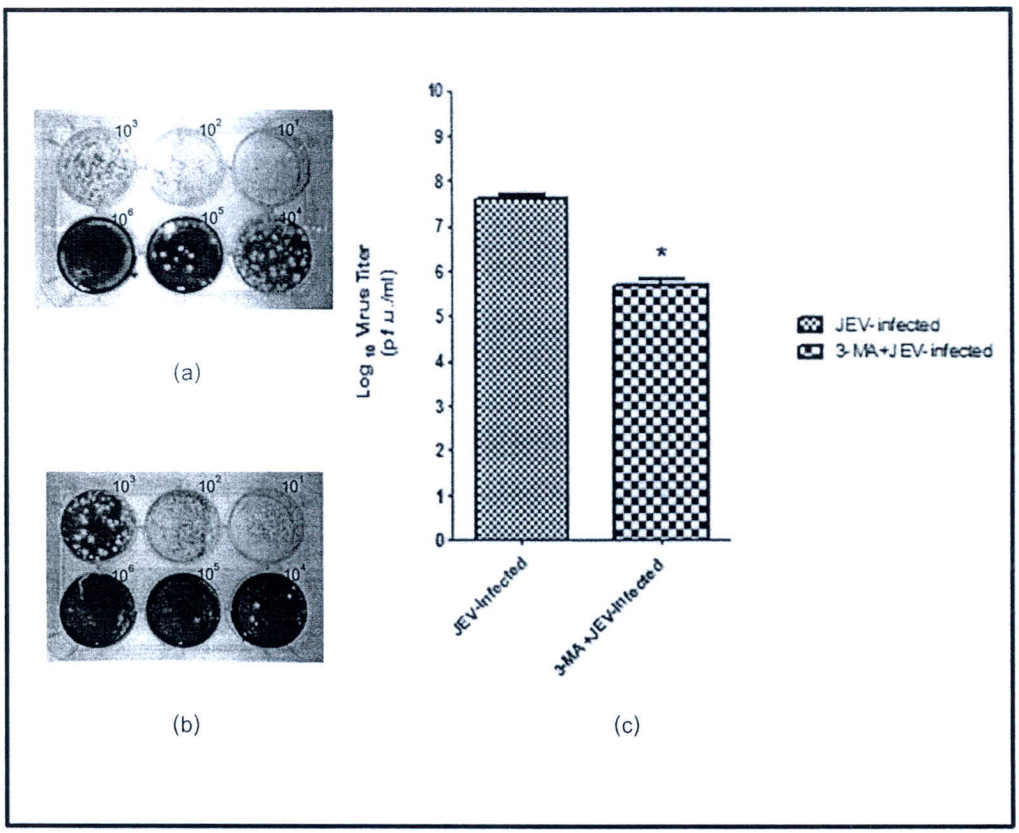

Figure 4.15 Viral production in JEV-infected CHME-5 cells in presence of autophagy inhibitor

This figure shows an effect of autophagy inhibitor (3-MA) on JEV production using standard plaque assay. The supernatants from JEV-infected CHME-5 cells with or without 3-MA pretreatment were collected at 48 hr. post infection for viral titer determination using standard plaque assay of JEV-infected CHME-5. **(a)** Standard plaque assay for viral production in JEV-infected CHME-5 cells (MOI of 100). **(b)** Standard plaque assay for viral production in JEV-infected CHME-5 cells (MOI of 100) with 10 mM 3-MA pretreatment. **(c)** Comparison of viral production shown as bar graph. The values represent the mean ± SEM from two independent experiments. (*, $p < 0.05$)