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INFECTIVITY AND CELL DEATH OF MICROGLIA IN RESPONSE TO JAPANESE ENCEPHALITIS VIRUS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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จิตสุมนต์ วัฒนะประเสริฐ : การติดเชื้อและการตายของเซลล์ไมโครเกลียจากเชื้อไวรัสไข้สมองอักเสบเจอี (INFECTIVITY AND CELL DEATH OF MICROGLIA IN RESPONSE TO JAPANESE ENCEPHALITIS VIRUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.ธนัญญา ทองตัน, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.ศุภางค์ มณีศรี เลอกรองด์, 85 หน้า.

ไวรัสไข้สมองอักเสบเจอีจัดอยู่ในกลุ่มแฟมิลี่ฟลาวิไวริเดอี ซึ่งพบได้บ่อยในแถบเอเชียและแปซิฟิค ถึงแม้ว่าเซลล์ที่เป็นเป้าหมายหลักในการติดเชื้อไวรัสคือเซลล์ประสาทในระบบประสาทส่วนกลาง เซลล์ไมโครเกลียก็สามารถถูกกระตุ้นและมีการหลั่งสารต่างๆเมื่อได้รับการติดเชื้อไวรัสเช่นกัน ปัจจุบันนี้มีรายงานเกี่ยวกับบทบาทของออโตฟาจีซึ่งเป็นกระบวนการที่เซลล์ใช้ย่อยสลายออร์แกเนลล์และโปรตีนที่เสื่อมสภาพที่สำคัญในสภาวะติดเชื้อไวรัส ดังนั้นงานวิจัยนี้จึงต้องการศึกษาความเกี่ยวข้องของออโตฟาจีของเซลล์เพาะเลี้ยงไมโครเกลีย (CHME-5) จากมนุษย์ในสภาวะติดเชื้อไวรัสไข้สมองอักเสบเจอี เมื่อเซลล์ได้รับเชื้อไวรัสไข้สมองอักเสบเจอีปริมาณ 10 เท่า พบว่าจำนวนเซลล์ทั้งหมดน้อยกว่าที่พบในเซลล์ควบคุมซึ่งไม่ได้รับการติดเชื้อเล็กน้อย อย่างไรก็ตามเมื่อเซลล์ได้รับเชื้อไวรัสไข้สมองอักเสบเจอีปริมาณ 100 เท่า พบว่าจำนวนเซลล์ทั้งหมดลดลงอย่างมีนัยสำคัญโดยสามารถสังเกตได้หลังการติดเชื้อในวันที่ 2 เป็นที่น่าสนใจว่าเปอร์เซนต์เซลล์ตายซึ่งตรวจสอบโดยวิธี trypan blue dye exclusion assay มีค่าใกล้เคียงกันไม่ว่าเซลล์จะได้รับเชื้อไวรัสไข้สมองอักเสบเจอีปริมาณ 10 เท่าหรือ 100 เท่า (ปริมาณเซลล์ตายน้อยกว่า 10%) จากผลการทดลองนี้บ่งชี้ถึงอัตราการแบ่งตัวเพิ่มจำนวนของเซลล์ที่ช้าลงเมื่อเซลล์เพาะเลี้ยงไมโครเกลียได้รับไวรัสในปริมาณที่สูงขึ้น ผลการศึกษาความสามารถในการติดเชื้อไวรัสไข้สมองอักเจอีด้วยเทคนิค immunocytochemistry พบอนุภาคไวรัสในไซโทพลาซึมของเซลล์ไมโครเกลียโดยมีเซลล์ที่ติดเชื้อไวรัสเท่ากับ 78% ในวันที่ 2 ของการได้รับเชื้อไวรัสไข้สมองอักเสบเจอี จากผลการศึกษากระบวนการออโตฟาจีด้วยกล้องจุลทรรศน์อีเลคตรอนแบบส่องผ่านพบถุงออโตฟาโกโซมที่มีอนุภาคของไวรัสบรรจุข้างใน นอกจากนี้เมื่อทำการตรวจสอบการแสดงออกของโปรตีน LC3-II ด้วยเทคนิค Western blottingและ indirect immunofluorescence ยังพบการแสดงออกของโปรตีนที่เพิ่มสูงขึ้นเมื่อเซลล์ติดเชื้อไวรัส สำหรับการตรวจสอบจำนวนไวรัสที่ผลิตขึ้นในสภาวะที่เซลล์ได้รับสารยับยั้งออโตฟาจี 3-methyladenine พบว่าจำนวนไวรัสผลผลิตลดลง ซึ่งสามารถเสนอแนะได้ว่าออโตฟาจีมีความเกี่ยวข้องกับการเพิ่มจำนวนของไวรัส จากผลการศึกษาทั้งหมดบ่งชี้ว่าเซลล์ไมโครเกลียของมนุษย์สามารถติดเชื้อไวรัสไข้สมองอักเสบเจอีและการติดเชื้อไวรัสไข้สมองอักเสบเจอีสามารถเหนี่ยวนำให้เกิดกระบวนการออโตฟาจี

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีทางการแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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JITSUMON WATTANAPRASERT : INFECTIVITY AND CELL DEATH OF MICROGLIA IN RESPONSE TO JAPANESE ENCEPHALITIS VIRUS. ADVISOR: ASST. PROF. THANANYA THONGTAN, Ph.D., CO-ADVISOR : ASST. PROF.SUPANG MANEESRI LE GRAND, Ph.D., 85 pp.

Japanese encephalitis virus (JEV), an arbovirus that belongs to the family *Flaviviridae*, is endemic to large parts of Asia and Pacific. Even though neurons have been proposed to be the principle JEV target cells in the central nervous system, microglia are activated and secreted many mediators in response to JEV infection. Recently, autophagy, a cellular process involved in the degradation of organelles and aggregated long-lived proteins, has been reported to play a significant role in viral infection. This study aimed to determine the involvement of autophagy in response to JEV infection in human microglial (CHME-5) cells. Upon JEV infection at a multiplicity of infection of 10, a small deficit in total cell number, compared to that of mock-infected CHME-5 cells was observed. However, with the multiplicity of infection of 100, a significant reduction in total cell number was observed since day 2 post infection. Interestingly, the percentage of cell death, determined by tryptan blue dye exclusion assay, was relatively the same (less than 10%) in both levels of multiplicity of infection, indicating a slower proliferation rate in JEV-infected microglial culture with a higher multiplicity of infection. By immunocytochemistry, JEV virions were clearly detected in cytoplasm of microglial cells, with an estimated of 78% infectivity at day 2 post infection. Electron micrographs demonstrated autophagosomes with JEV virions. Besides, an increase in the expression of LC3-II in response to JEV infection was detected by indirect immunofluorescence and western blotting. In addition, inhibition of autophagy, using 3-methyladenine, led to a reduction of the JEV production suggesting autophagy mediated viral replication. Collectively, these results indicate that human microglia are susceptible to JEV and autophagy is induced during JEV infection.

Department : BIOCHEMISTRY Student's Signature .....

Field of Study : MEDICAL BIOCHEMISTRY Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

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List of Abbreviations

%	Percent
µg	Microgram
µl	Microlitre
µm	Micrometre
°C	Degree Celsius
$CaCl_2$	Calcium Chloride
DAB	3,3-Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
E protein	Envelope Protein
EBSS	Earle's Balanced Salt Solutions
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
g	Gram
H_2O	Water
hr(s)	Hour(s)
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
JE	Japanese Encephalitis
JEV	Japanese Encephalitis Virus
KCl	Potassium Chloride
kDa	Kilodalton
LAMP-1	Lysosomal-associated membrane protein 1
LC3	Microtubule-associated 1 light chain 3

M	Molar
3-MA	3-Methyladenine
mA	miliampere
MEM-α	α-modified Minimal Essential Medium
mg	Milligram (s)
$MgSO_4$	Magnesium Sulphate
min	Minute (s)
ml	Milliliter
MOI	Multiplicity of Infection
MW	Molecular Weight
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Hydrogen Carbonate
nm	nanometre
NS	Nonstructural Protein
NS1	Nonstructural Protein 1
NS2A	Nonstructural Protein 2A
NS2B	Nonstructural Protein 2B
NS3	Nonstructural Protein 3
NS4A	Nonstructural Protein 4A
NS4B	Nonstructural Protein 4B
NS5	Nonstructural Protein 5
p.f.u.	Plaque-Forming Unit
PBS	Phosphate Buffered Saline
rpm	Revolution per Minute
SDS	Sodium Dodecyl Sulfate
Seakem LE agarose	Seakem Low Electroendosmosis Agarose
TEM	Transmission electron microscope

Tris base	2-Amino-2-(Hydroxymethyl)-1,3-Propanediol
TRITC	Tetramethylrhodamine-5-(and 6)-isothiocyanate
V	Volt
YE-LAH	Yeast Extract-Lactalbumin Hydrolysate

CHAPTER I

INTRODUCTION

Background and rational

Japanese encephalitis (JE) is one of the most endemic viral encephalitis in East and South East Asia with an estimated 50,000 cases annually. The mortality rate is approximately 20-30% (1, 2). Japanese encephalitis virus (JEV), a positive single stranded RNA arbovirus, belongs to family of *Flaviviridae*. Clinical manifestations of JE patients are ranging from non-specific flu-like illness to acute and possibly fatal encephalitis. Most of the survivors have neurological sequelae such as mental retardation and epilepsy. The severity of the disease depends on the location and the degree of brain destruction. However, pathogenesis of Japanese encephalitis remains unclear (2-4).

During JEV infection, even though neuron performs as a primary target cell in central nervous system (CNS) but microglia, a brain residental macrophage, is activated by JEV infection(5). The JEV antigens have been detected in neuronal and glial cells (6, 7). The activation of microglia demonstrates, at microscopic level, a common histological feature in both of human autopsy and animal experimental models (8).The mechanism of neuronal cell death upon JEV infection is proposed in two ways. One is direct neuronal killing, the viral multiplications within neuronal cells cause cell death. The other is indirect mode of killing, whereas massive inflammatory response causes an up-regulation of reactive oxygen species and pro-inflammatory cytokines (9). It had been reported that many mediators like IFN-α, TNF-α,MIF, IL-8, IL-6, RANTES, IL-1ß and MCP-1 are elevated during JEV infection (9-11).

In recent study, microglial were suggested as a viral reservoir for CNS infection and JEV infection also triggered apoptosis (12). The overexpression of Bcl-2, antiapoptotic gene, can effectively delay JEV-induced cell death and consequently convert some target cells such as Baby hamster kidney (BHK-21) and Chinese hamster ovary (CHO) cells into persistently infected cells (13, 14). Infection of JEV triggers initiators caspase-8 and -9, probably through FADD-independent but mitochondrion-dependent path ways in mouse neuroblastoma (N18) cells (15).

Besides apoptotic cell death, autophagy can commonly be found in virus infected cells. The autophagy is the cellular degradation pathway for long-lived proteins, aggregated

proteins and damage organelles that involved delivery of cytoplasmic cargoes to lysosomes. Autophagy is induced during cell differentiation, starvation and intracellular stress. Several viruses trigger autophagy upon their infection such as Herpes simplex virus, Polio virus, Dengue virus (16, 17).

As there is currently no information about autophagy upon JEV infection in human microglial cell, therefore, this research aims to examine, whether Japanese encephalitis virus infection can trigger autophagic in human microglial cell death or not. The results from this study will provide basic knowledge for understanding JE pathogenesis.

Research Questions

Does Japanese encephalitis virus infection induce autophagic microglial cell death?

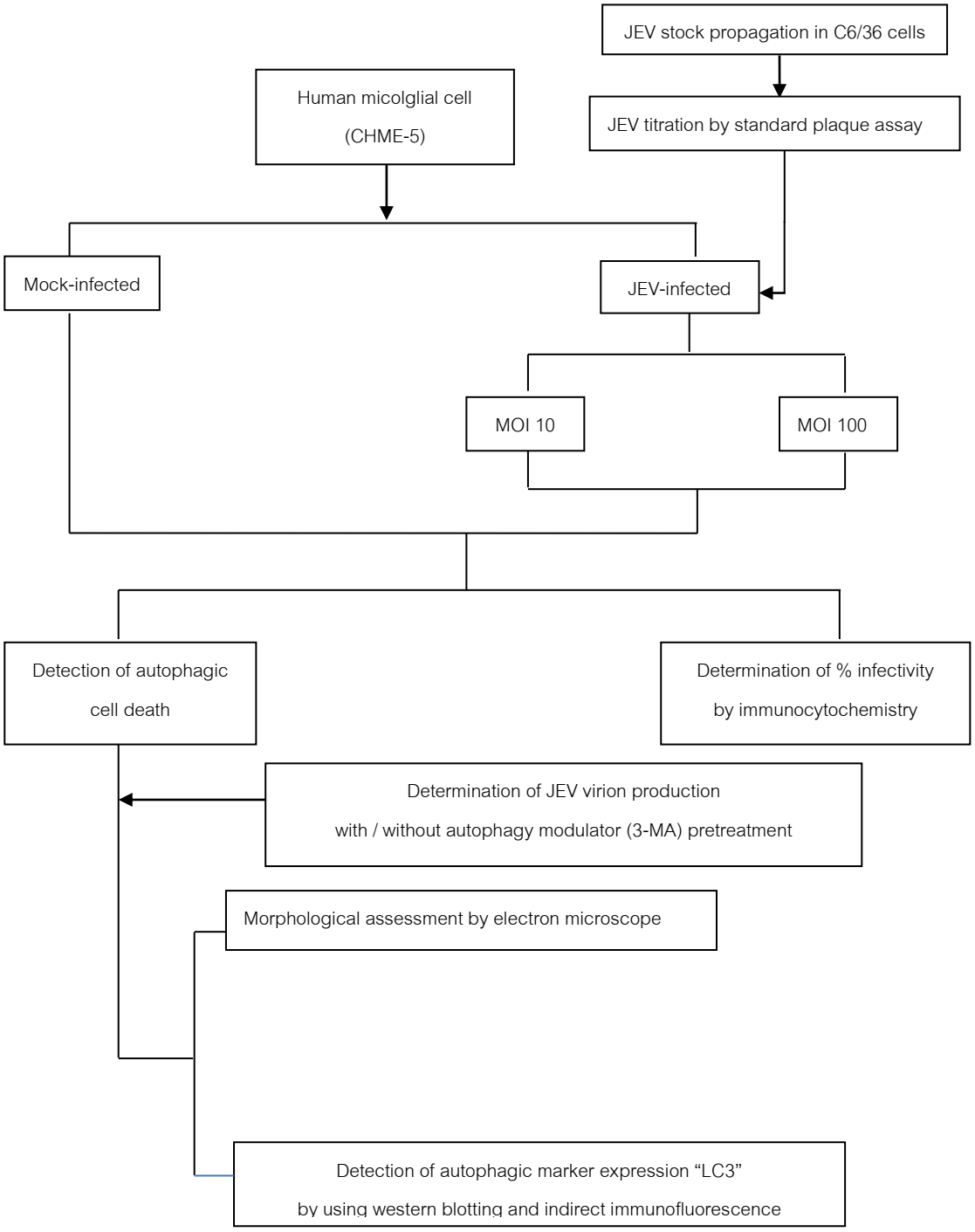
Objectives

1. To examine the autophagic cell death in human microglial cell in response to JEV infection.
2. To determine the percentage of infectivity of JEV in human microglial cell.

Hypothesis

Japanese encephalitis virus infection cause autophagic microglial cell death.

Conceptual Framework



Assumption

All Instruments were tested for the precision and accuracy according to the corresponding standardizations.

Keywords;

Japanese encephalitis virus

Microglia

Autophagy

Ultrastructure

Operational Definitions

Plaque assay is a method used to determine viral titration as plaque forming units (p.f.u.)/ml. A viral plaque is formed when a virus infects a cell. The infected cell would be seen as a white plaque for counting and calculating the virus titer, after crystal violet dye staining.

MOI stands for "Multiplicity of Infection". It is the ratio of virions to the number of cells being infected.

Percentage of the infectivity

$$\text{Percentage of the infectivity} = \frac{\text{Number of infected cells}}{\text{Total number of cells}} \times 100$$

Percentage of punctuate staining

$$\text{Percentage of punctuate staining} = \frac{\text{Number of positive punctuate staining cells}}{\text{Total number of cells}} \times 100$$

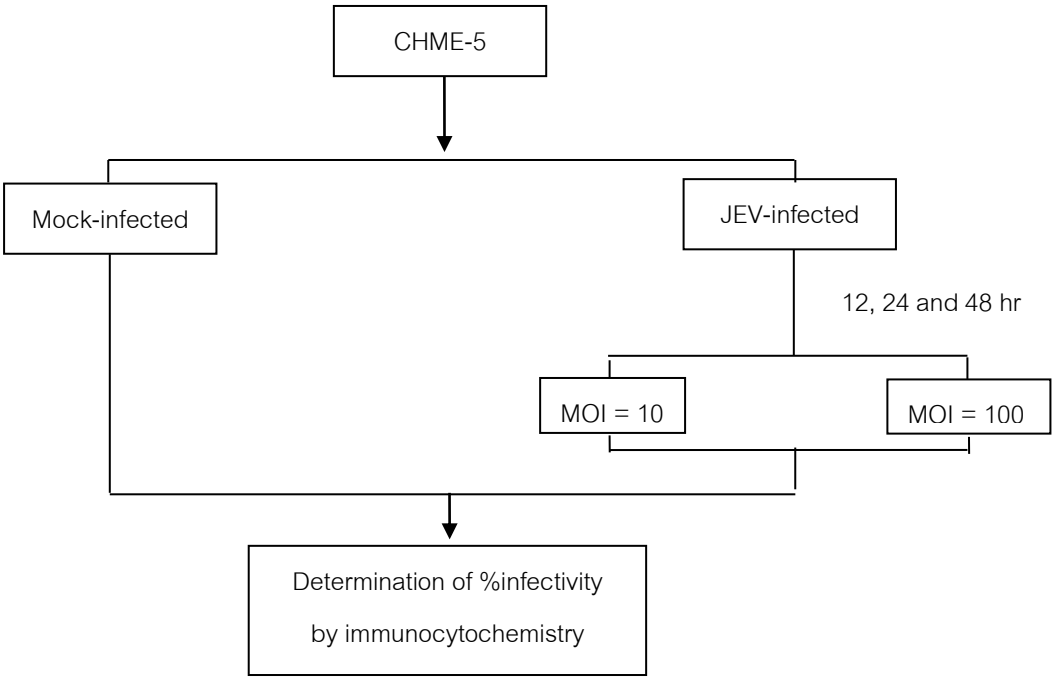
Research Design

Experimental Research

Research Methodology

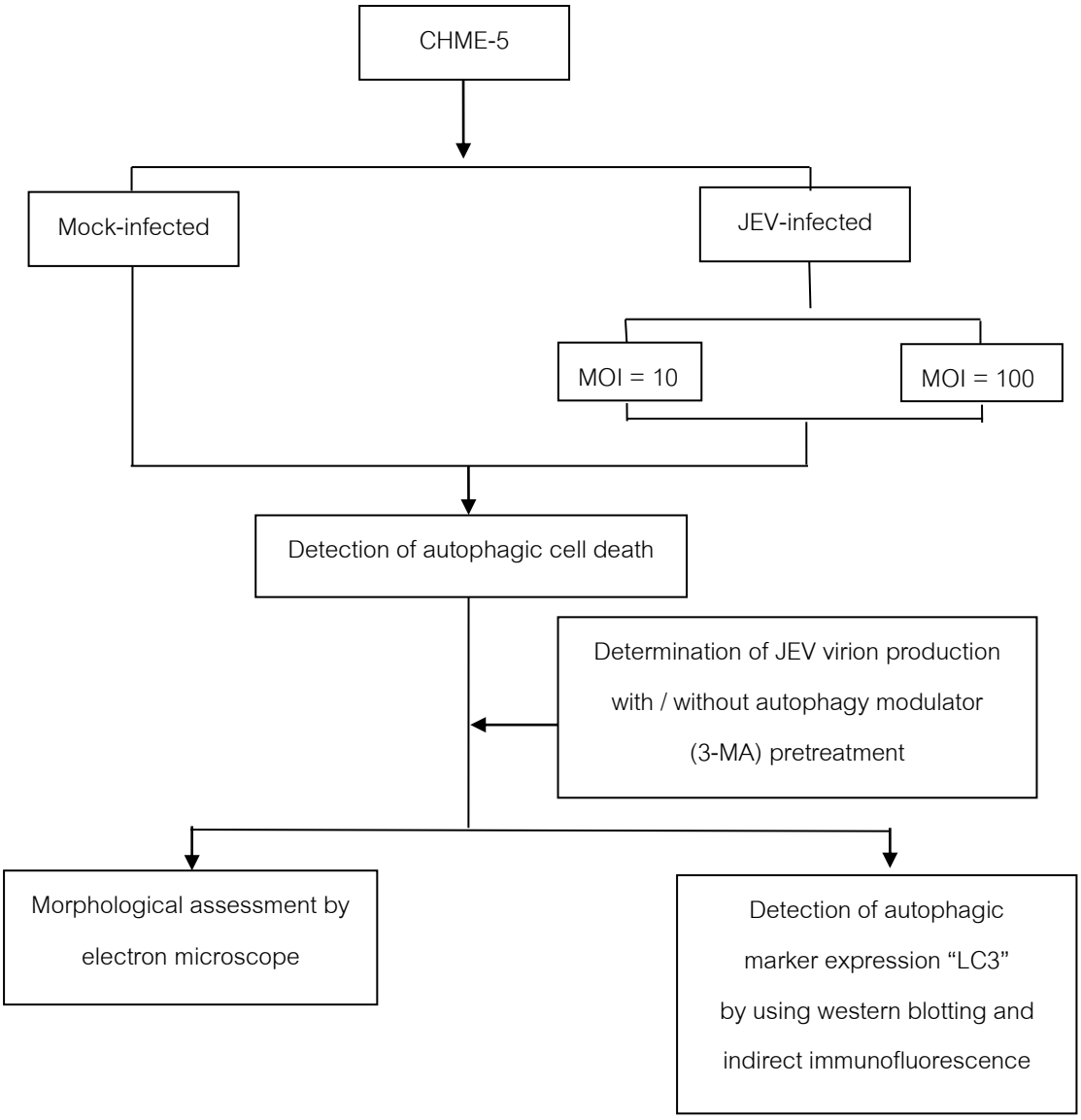
Experimental I: Determination of the percentage of infectivity in JEV-infected CHME-5 cells.

To determine the condition with, at least, an estimated of 70% infectivity for autophagic cell death examination, the cells were seeded onto glass cover slips and divided into two groups as follows: control and JEV- infected group. Control group were treated with normal culture media. In JEV infected group, cells were directly infected for 2 hr with JEV at an MOI of 10 and 100. Then, cells were cultured for 12, 24, 48 hr. Cells were later be processed for immunocytochemistry detection using anti-flavivirus as a primary antibody.



Experiment II: The autophagy assessment in JEV-infected CHME-5 cells.

CHME-5 cells were either mock-infected or JEV-infected for 2 hr with JEV at an MOI of 10 and 100. Then, cells were cultured for 48 hr and processed for morphological assessment by Transmission electron microscope. For detection of autophagic marker, LC3, cells were processed for indirect immunofluorescence and western blotting by using anti-LC3B as a primary antibody. To determine JEV viral production pretreats with/without autophagy modulator (3-MA), culture media were collected at 48 hr and measured virus titers by standard plaque assay.



Benefits of study

1. Better understanding of the Japanese encephalitis pathogenesis
2. To find out the role of autophagic cell death in microglia in which might be applied to other arboviral encephalitis
3. To provide basic knowledge for the development of Japanese encephalitis antiviral drugs

Obstacles and Strategies to Solve the Problem

1. Cell culture contamination: All kind of procedures involving cell culture require aseptic techniques.
2. Cell line cross-contamination: This research uses many cell types. Each cell line should be sub-cultured separately.

CHAPTER II
REVIEW LITERATURES

2.1 Japanese encephalitis virus

2.1.1 General Background

Japanese encephalitis virus (JEV) is a mosquito-borne virus classified in family *Flaviviridae* genus *Flavivirus.* The virus consists of 5 genotypes, and it is transmitted to human by the bite of an infected mosquito. The major vector is *Culex tritaeniorhychus*. Infection by JEV leads to a wide spectrum of clinical presentation from a febrile headache to acute and possibly fetal encephalitis with 20-30% mortality rates (1, 2). Most of survivors have neuronal sequelae such as mental retardation, epilepsy, paralysis, deafness and blindness. At present, specific treatments and antiviral drug for JEV infection are not available. Early diagnosis and supportive treatment helps to reduce the mortality rate of JEV infection (2, 3).

2.1.1.1 Epidemiology

Japanese encephalitis virus was firstly isolated in Japan in 1935 but had been found in the area as early as 1870 (18). Japanese encephalitis (JE) affects more than 50,000 patients and 15,000 deaths annually (3). From there, JE had been spread to many countries especially in Eastern and Southeastern Asia such as China, Siberia, Korea, Japan, Taiwan, Guam, Saipan, Vietnam, Cambodia, Thailand, India, Nepal and Sri Lanka. Moreover, it has increasingly been found throughout most countries of East and Southeast Asia (Figure 2.1). The factors that support disease in this area comprise of the vector, the environmental conditions which are essential for mosquito breeding cycle such as rainfall, humidity, tropical temperatures, and the number of the amplifying host that are pigs and birds. Furthermore, JE spreading also related to rapid globalization and change in global climatic condition due to industrialization and deforestation (1-3, 19).

In Thailand, the first epidemic of JEV was reported in Chiang mai and nearby areas in 1969. Later on, there was a study of JEV genotype distribution in Thailand by collecting data in 7 provinces such as Chiang mai, Khon Khen, Nakhon Pathom, Ratchaburi, and Phuket. These provinces represent 4 parts of Thailand including North, North-east, Midland and South. Increasing of pig farming and trading is relevant to the dispersion of JEV (20).

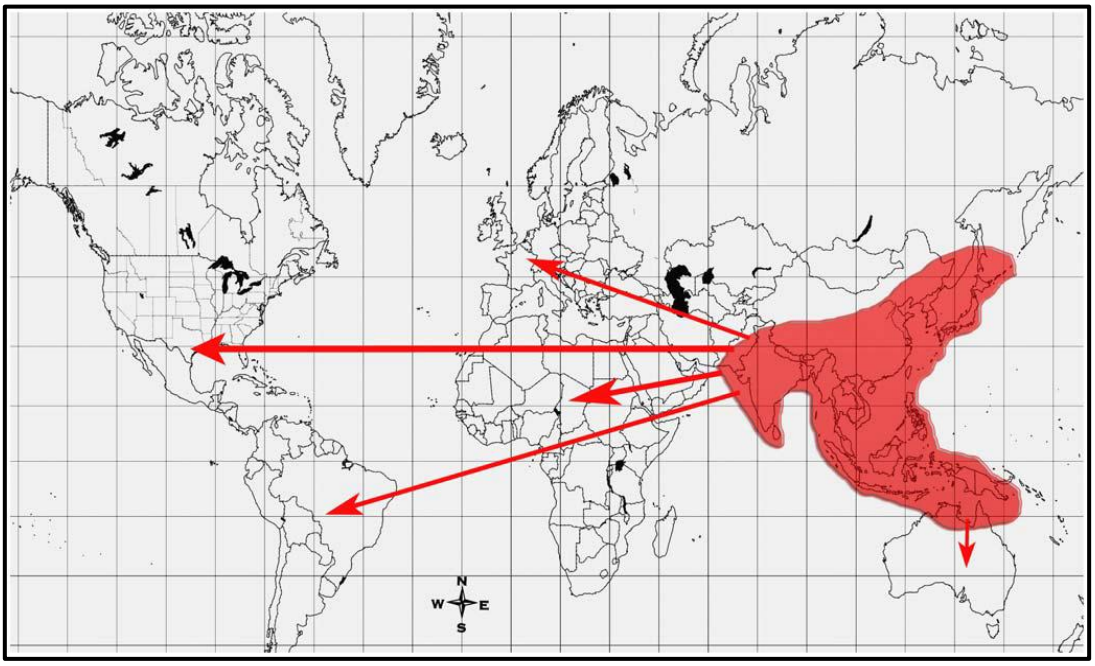


Figure 2.1 The global distribution of JEV (3)

This fiqure shows the global distribution of JEV. The red shaded areas indicate the present distribution of JEV, whereas the arrows indicate the areas where there is a high possibility of virus spread due to globalization and climatic change (3).

2.1.1.2 Clinical manifestations

After Japanese encephalitis virus (JEV) has invaded to human body by a mosquito's bite. The incubation period is 5-15 day. Clinical presentations in JE patients depend on the part of nervous system that is affected. Most of JEV infections are either asymptomatic or may result in a nonspecific flu-like illness follow by symptomatic such as reduced level of consciousness, seizure, headache, photophobia and vomiting. Some patients present with aseptic meningitis or acute flaccid paralysis. Severe form of JE patients is associated with respiratory paralysis and encephalitis. Survivors from JEV infection also have neurological sequelae such as epilepsy, paralysis and deafness. Furthermore, the extrapyramidal features include pill-rolling movement, opsoclonus myoclonus and bizarre facial grimacing, staring mask-like faces and lip smacking can be found in survivor patient (Figure 2.2) (2, 3, 21, 22).

2.1.1.3 Transmission cycle

JEV is transmitted to humans by a bite of infected mosquito vector, especially *Culex tritaeniorhynchus*. Pigs and warding birds are amplifying hosts. Humans are dead-end hosts in JEV transmission cycle because of low levels of viremia. Other domesticated animals such as dogs, sheep and rodent may become infected, however fail to develop a sufficient viremia for further viral amplification (Figure 2.3) (2, 3, 23).

2.1.1.4 Treatment

Up to date, no specific antiviral agent to mitigate the effects of JEV is available. JE therapy consists of supportive care and management of complications (3, 24).

Minocycline, a board spectrum synthetic antibiotic classified in tetracycline group, were suggested as an anti-JEV drug. Kumar et al. investigated that minocycline treatment can reduce neuronal apoptosis, microglia activation including caspase activity (25).

2.1.2 Classification and molecular biology

Japanese encephalitis virus belongs to the family *Flaviviridae*, genus *Flavivirus*. Apart from JEV, other members of the family can also cause encephalitis (Figure 2.4). West Nile Virus causes encephalitis and neuronal disorders in Africa, Southern and Central Europe, India, the Middle East and North America and St. Louis encephalitis virus is a major cause of viral encephalitis in North and South America (21, 26) .

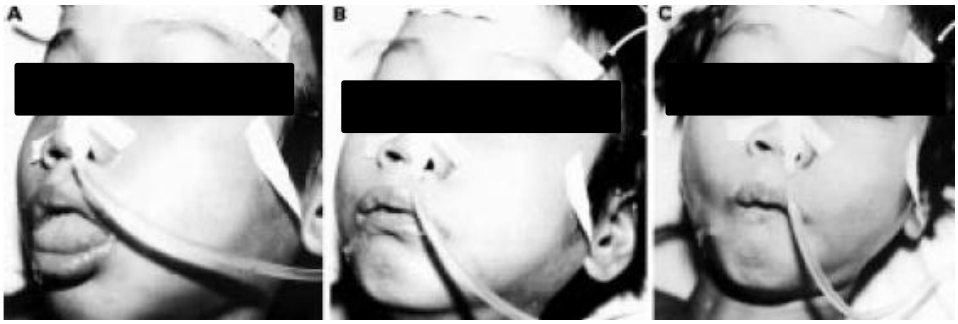


Figure 2.2 Facial grimacing in a Vietnamese boy with Japanese encephalitis (21).

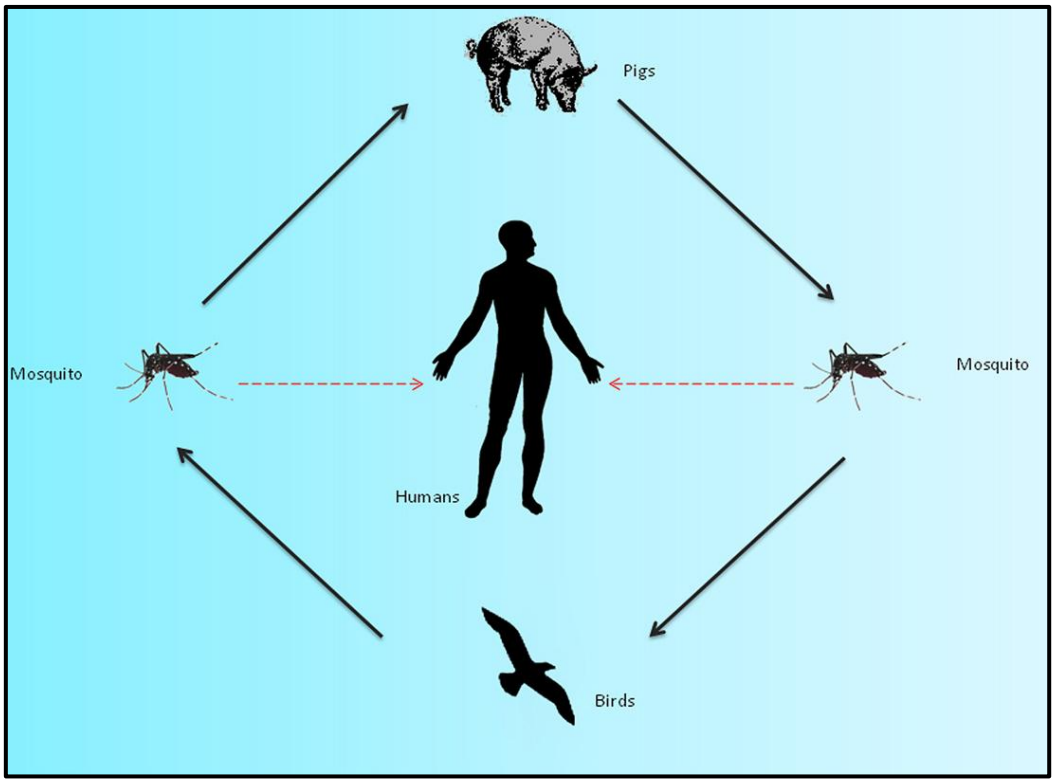


Figure 2.3 The transmission cycle of Japanese encephalitis virus (23)

This figure demonstrates the Japanese encephalitis virus transmission cycle. The virus transmitted in an enzootic cycle among birds, pigs and other vertebrate host through a bite of mosquitoes. Human is dead end host for the JEV (23).

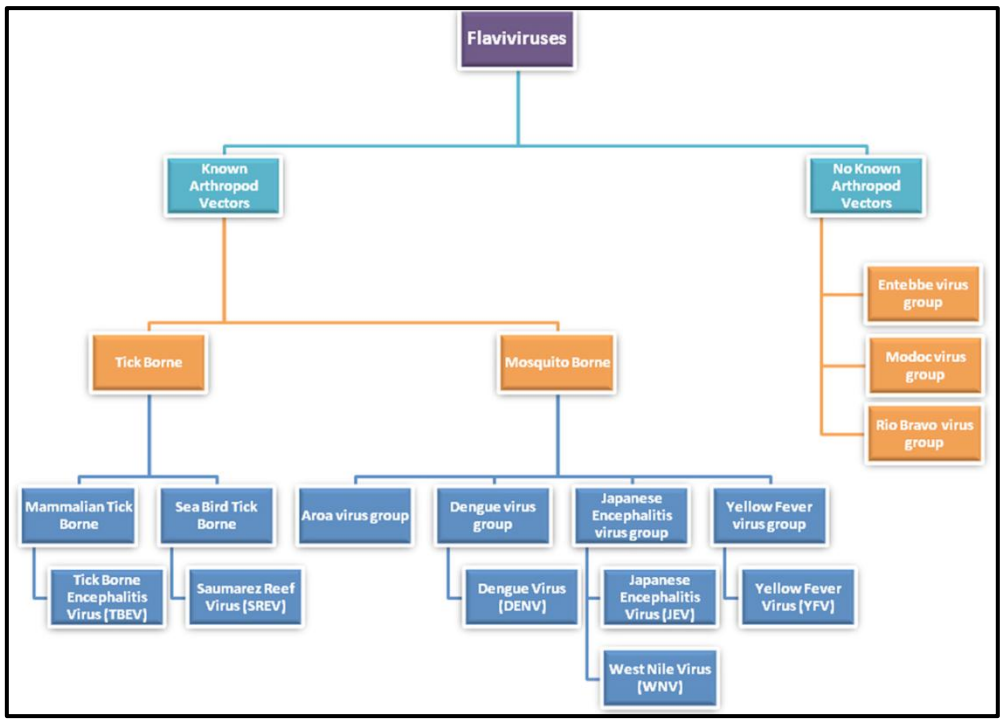


Figure 2.4 The classification of *Flavivirus* (23)

This figure show the classifications of *Flavivirus* . Japanese encephalitis virus can be classified as a mosquito-borne virus as same as West Nile virus, Dengue virus and Yellow fever virus (23).

There are at least 5 genotypes that are isolated from different geographic areas using nucleotide sequencing of C/PrM and E genes. Genotype I were isolated from Northern Thailand, Cambodia and Korea. Genotype II were isolated from Southern Thailand, Indonesia, Malaysia and Australia. Genotype III were isolated from many areas of Asia such as Japan, Korea, China, Taiwan, Philippines, India and Sri Lanka and Nepal. Genotype IV were isolated from Indonesia and Genotype V were isolated from some part of Malaysia and Singapore (21).

Japanese encephalitis virus is an enveloped virus, which consists of a 11 kb single stranded RNA viral genome. The RNA comprises of 5'- and 3'-untranslated regions (UTRs) and a single open reading frame encodes three structural proteins (**C**apsid protein, **Pr**ecursor to the **M**embrane protein, **E**nvelope protein) and seven nonstructural proteins)NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The E protein is a large structural protein that is important for virus entering into the host cell and it is a major target of host immune response (Figure 2.5) (22, 23, 27).

2.1.3 Life cycle and replication

Entry of JEV to host cells begins with attachment of virus particles onto the cell surface. JEV is internalized into the host call by receptor-mediated endocytosis and then virus is transported to early endosome that subsequently mature to late endosomes. The low pH environment in endosome triggers major conformation changes in the virion. This structure rearrangement mediates fusion of viral and cellular membranes. Lack of prM cleavage during maturation prevents conformation change of E protein during fusion. After the fusion the nucleocapsid is released into cytoplasm and translation of viral RNA is initiated (27).

Translation of viral RNA genome occurs in one open reading frame to from a viral polyprotein that is processed by host cell and viral protease. The structural proteins and non-structural protein are synthesized. Following the viral protein production, NS protein, the viral RNA genome and probably host factors are assembled to form the replication complex. Replication process occurs in association with membrane structure presumably by interaction involving hydrophobicity of some NS proteins. The replication of viral RNA occurs on intracellular membrane. Virus assembly occurs on the surface of rough endoplasmic reticulum (ER). The immature particles are transported to the secretory pathway of the trans-Golgi network (TGN), prM is cleaved by furin. Mature virions are then released into the cytoplasm by exocytosis (23, 28).

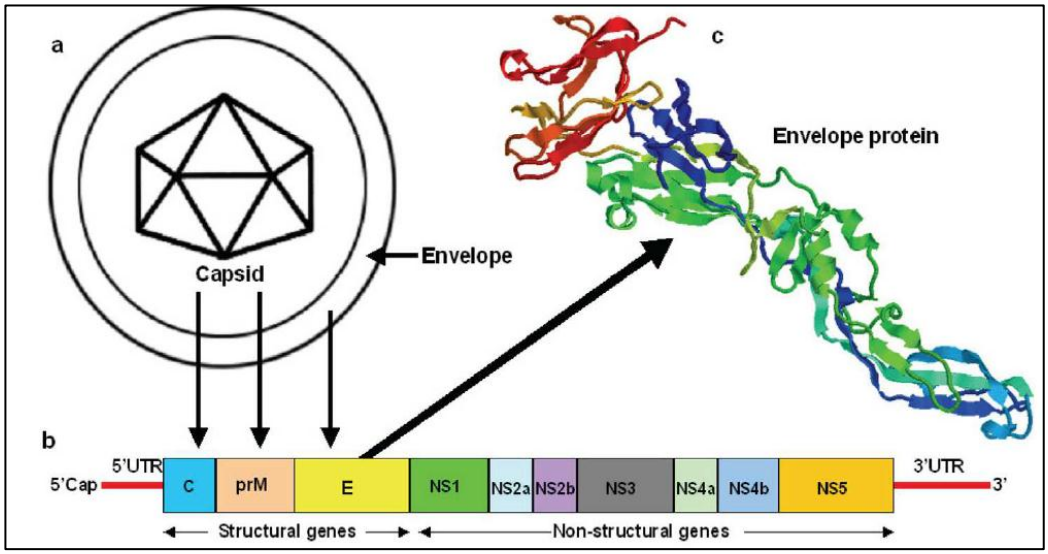


Figure 2.5 Schematic of Japanese encephalitis virus structure (19)

This figure demonstrates the schematic of Japanese encephalitis virus structure. **(a)** The JEV virion has a diameter of 50 nm. The envelope consists of E and M protein. The icosahedral nucleocapsid is composed of RNA viral genome and C protein. **(b)** Diagram of JEV genome indicates the orientation of genes encoding viral proteins. **(c)** Structure of E protein homodimers (19).

2.2 Microglia and JEV infection

Microglia are resident immune cells of the CNS and have an important role in host defense against invading microorganism. Microglia constitute 10-15% of cells in the CNS. Normally, microglia can be found in healthy mature CNS especially in hippocampus section. At the resting stage, microglia has a characteristic as ramified morphology (Figure 2.6).

Upon the activation by infection, trauma, neurogenerative disease or altered activity that is bothered brain homeostasis, microglia become amoeboid shape and migrate to the site of injury where they proliferate and release pro-inflammatory cytokines (28-30).

The mechanism by which JEV causes neurological disease remains unclear. Many studies have delineated important pathways being triggered in the process. RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) is one of an important chemokine that has been shown to be secreted by the microglia and astrocytes upon JEV infection. The expression of this chemokine recruited other immune cells to the area of infection (31). Ghoshal et al. showed that the number of activated microglia significantly elevated upon JEV infection. Activated microglial cells cause an up-regulation of reactive oxygen species and pro-inflammatory cytokines such as MCP-1, IFN-α, TNF-α, IL-8, and IL-6 (10, 32). Recent studies demonstrate that neuronal death occurs as a result of the inflammatory response, initiated during JEV infection (10, 25, 33). Moreover, JEV infection in animals as well as in vitro also results in the induction of pro-inflammatory cytokines, IL-1β and IL-18 (34). Previous study by Thongtan et al. reported that mouse microglial cells might serve as reservoir of JEV and JEV infection induced apoptotic cell death in microglia (12). Furthermore, Nazmi and colleagues investigated that JEV-infected peripheral macrophage can disseminate to blood brain barrier and possibly served as “Trojan Horses” to introduce virus to CNS and released pro-inflammatory cytokines which lead to apoptotic cell death of neurons (11).

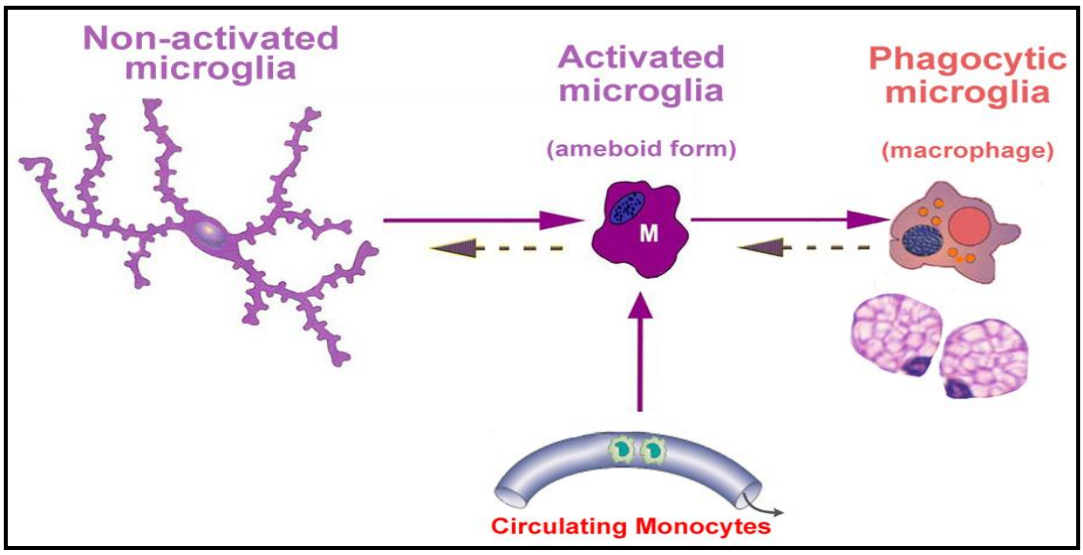


Figure 2.6 Morphology of microglia (35)

This figure shows the morphological change of microglia. At the resting stage, microglial cell has a characteristic as ramified morphology. With the activation, microglia convert to amoeboid forms and migrate to the site of injury, which show macrophage properties as a phagocytic cell.

(http://missinglink.ucsf.edu/lm/introductionneuropathology/Response%20_to_Injury/Microglia.htm)

2.3 Autophagy

Autophagy is the process of physiologically and immunologically controlled that sequesters and degrades cytoplasmic targets, including soluble macromolecular aggregates, lipids, and nucleotides from damaged proteins, cellular organelles, and intracellular pathogens such as, bacteria, protozoans and viruses (36). Autophagy is constantly maintained at the basal level and is up-regulated in response to stress conditions. There are at least three different types of autophagy classified by the route of delivery to lysosome, macroautophagy, microautophagy, and chaperone-mediated autophagy.

1. Macroautophagy, a major type of autophagy, cytoplasmic contents are delivered to lysosome with the formation of a double-membrane structure called the autophagosome which sequesters cytosolic material and delivers to the lysosome for degradation. Macroautophagy is conserved in mammals, and is mediated by a special organelle termed the autophagosome.
2. Microautophagy is the transfer of cytosolic components into the lysosome membrane itself by non-selectively engulfs a cytoplasm by invagination of the lysosomal membrane.
3. Chaperone-mediated autophagy (CMA) is characterized by their selective degradation. Cytosolic protein containing KFERQ –like motifs are recognized by a cytosolic Hsc70 that binds to lysosomal-associate membrane protein-2a (LAMP-2a) (37-39).

However, in most studies, the word “Autophagy” usually refers to Macroautophagy as its used here.

2.3.1 Molecular events of autophagy

Autophagy is a general process used for degradation. It is activated at the basal levels in most cell types in order to balance of organelles, protein and homeostasis of macromolecules. The process of autophagy involves a set of evolutionarily conserved gene products, known as Atg proteins including Atg 4, 5, 7, 8, 10, 12 and 16, which are required for the formation of isolation membrane and autophagosome(36, 40-42). The process of an autophagic pathway composes of four major stages (Figure 2.7).

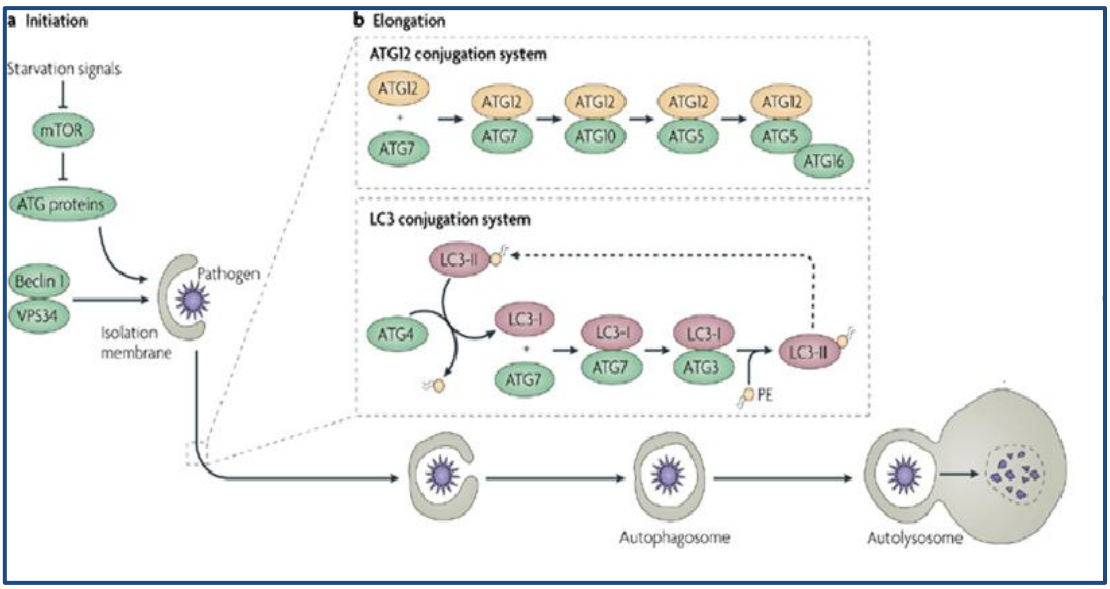


Figure 2.7 The Autophagy pathway (41)

(a) Initiation process of autophagy pathway is regulated by 2 regulatory molecules including mTor and Vps 34. **(b)** For elongation process or autophagosome formation needs two ubiquitin-like conjugation processes; Atg5-Atg12 conjugation system and lipid conjugation of Atg8 or LC3 conjugation system. For Atg12 conjugation system, Atg12 is activated by Atg7 which works as an E1-like ubiquitin activating enzyme, and then passed to Atg10 as an E2-like ubiquitin carrier protein to form linkage between Atg 12 and Atg 5. For LC3 conjugation system, LC3-I, cytosolic form is activated by Atg 7 and passed to E2- like enzyme Atg3 to form a covalent linkage between LC3 and phosphatidylethanolamine (PE), resulting in a conversion of LC3-I to membrane bounded form or LC3-II. PE molecules are eliminated from LC3-II by Atg4 for recycling (41).

2.3.1.1 Induction

As noted that, autophagy is regulated by set of Atg proteins. The autophagy regulatory molecules are the Ser/Thr kinase target of rapamycin (Tor kinase) and the class III phosphatidylinositol 3 kinase vesicular protein sorting 34 (Vps 34), the only PI3K that is evolutionarily conserved from yeast to mammals. Vps 34 binds to Atg6 or the mammalian homolog Beclin-1 to form a complex. In normal conditions with growth factors and abundant nutrients, mTOR activation is the major inhibitory signal that repress autophagy. Moreover, Bcl-2 can negatively regulate the activation of autophagy by interfering the Vps34-Bectin-1 complex. In contrast, during starvation, inhibition of Tor protein kinase leads to an induction of autophagy. Also, Bcl-2 is phosphorylated by Jnk1 such that it can no longer interact with Beclin-1. Some of biochemical agents that have an effect on Tor and Vps34 are usually used to regulate autophagy. Rapamycin acts as an autophagy induction by inhibit Tor kinase activity. On the other hand, wormannin and 3-methyladenine (3-MA) act as autophagy inhibitors by inhibiting class III PI3K kinase which is required for the early stages of autophagosome development (37, 41-43).

2.3.1.2 Acquisition of phagophore membrane

In this step, the cytoplasmic constituents are sequestered by unique membrane as known as phagophore. Membrane dynamic during this event is required ubiquitin-like conjugation systems which essential and also connected to elongation and autophagosome formation step. There are three enzymes which involved this process of conjugation: E-1 activating enzyme, E2-conjugating enzyme and E3-ligase enzyme. Firstly, Atg12 is activated by Atg7 which works as an E1-like ubiquitin activating enzyme and then transferred to E-2 like enzyme Atg10. Then, E2-like ubiquitin carried proteins to form covalently linkage between Atg 12 and specific lysine of Atg 5. After that, Atg5-Atg12 complexes bind to Atg16 to form Atg12-Atg5/Atg16 complex which is leading to induce a curve of the phagophore (17, 41, 44).

2.3.1.3 Elongation and autophagosome formation

This step is characterized by an increase in size of the phagophore, which wraps around the cytosolic component. The progression of autophagy also depends on another conjugating system: the formation of Atg8/PE conjugate. LC3 (microtubule-associated protein 1 light chain 3), a mammalian homolg of yeast Atg8, is normally expressed as a cytosolic protein and subsequently cleaved by Atg4 to form LC3-I. Then, LC3-I is activated by

E1-like enzyme Atg7 and transferred to E2-like enzyme Atg3 to form a covalent linkage between LC3-I and phosphatidylethanolamine. Lipidation of LC3-I results in the membrane bound form of LC3 (LC3-II) which located in autophagic membrane.

2.3.1.4 Lysosomal fusion and degradation

Upon this phase, the autophagosome directly fuses with the lysosomes or firstly fuses with endosome to form amphisomes, followed by lysosomal fusion to form autophagolysosome. After the fusion, macromolecules and sequestered material in autophagosome is released in the lysosomal lumen. Intra-lysosomal degradation occurs by lysosomal acid hydrolases to break the inner membrane of autolysosome (Figure 2.8) (17, 42, 47) (17, 41, 44).

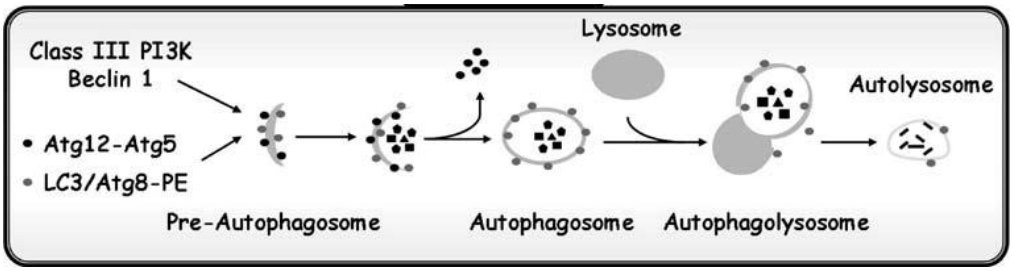


Figure 2.8 The autophagic process (17)

The autophagic process begins firstly with the nucleation of membrane structure called isolation membrane or phagophore. Secondly, **autophagosome formation**, cytosolic proteins and organelles are sequestered by a double membrane vesicle. Thirdly, **autophagosome fusion**, upon this phase, the autophagosome fuses with the lysosome to form autophagolysosome and finally a**utolysosome breakdown** (17, 41, 43).

2.3.2 Dection of autophagic cell death

There are three principal techniques for demonstrating autophagy as follow.

2.3.2.1 Electron Microscopy

The electron microscopy is the conventional method to observe a double-membrane vesicle defined as an autophagosome at the ultrastructural level. In the past, electron microscopy has been used extensively to quantify the number of autophagosomes in a variety of cells and tissues. Autophagosome is recognized as a double membrane structure containing undigested cytoplasmic materials including organelles, while the autolysosome is a single membrane structure containing cytoplasmic components at various stages of degradation. These structures are often generalized as the autophagic vacuoles (Figure 2.9). To date, electron microscope is a very informative method for monitoring autophagy, however, it should be complemented by additional assay to ensure correct identification and quantification (45-47).

2.3.2.2 Fluorescence Microscopy

The assessment of autophagosome number by electron microscopy requires considerably specialized expertise, and is becoming increasingly replaced by light microscopy and biochemical methods that are more widely accessible to researchers in a variety of fields. As mentioned above, the mammalian autophagy protein, LC3 is a universal marker of autophagosomes. This protein is modified by a ubiquitin-like conjugation during autophagy induction (37, 38). Modification of LC3 from cytosolic from (LC3-I) to membrane bound form (LC3-II) can be monitored as punctuate dots. The number of punctuate LC3 can be visually count under fluorescence microscopy (42, 46, 48, 49).

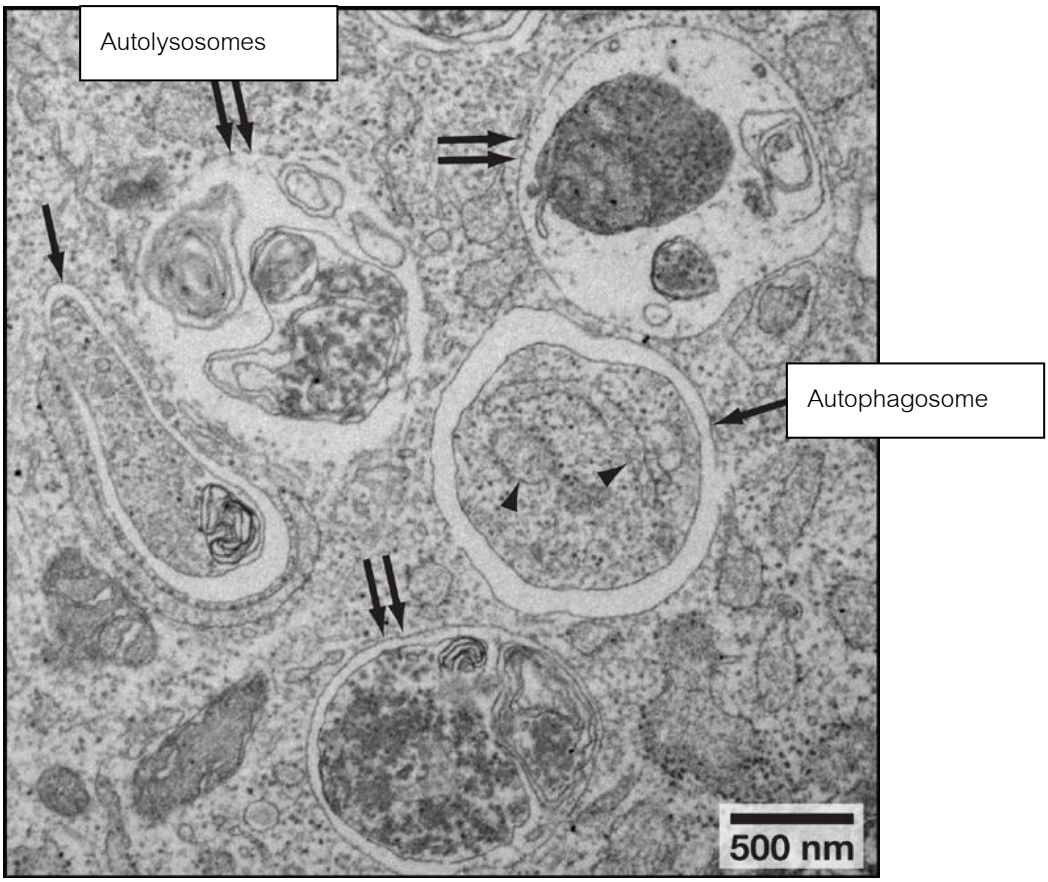


Figure 2.9 The Electron micrograph of autophagosome and autolysosome in starved mouse embryonic fibroblast cells (42)

This figure show morphological assessment of autophagosome and autolysosome in mouse embryonic fibroblast cells. The single arrows indicate autophagosome and double arrows indicate autolysosome which consist of degradation organelles (42).

Moreover, the co-localization of LC3 and protein markers which identify other cellular structure such as endosomes and lysosomes helps to identify stages of autophagic vacuole maturation. For example, co-localization of LC3 with LAMP-1 (lysosomal-associated membrane protein 1) indicates fusion of autophagosome with endosome or lysosome which result in amphisome and autophagolysosome respectively (50).

2.3.2.3 Immunoblotting

Besides its application in fluorescence microscopy assays, LC3 is also useful in biochemical assays to assess autophagy. The conversion from endogenous LC3-I to LC3-II can be detected by using immunoblotting with antibodies against LC3. Apparent molecular weight of LC-I on SDS-PAGE is 16 kD while that of LC3-II is 14 kD. The amount of LC3-II or the comparison of LC3-II/internal control ratio correlates with the amount of autophagosomes which reflects autophagic activity (45, 51).

2.3.3 Autophagy and virus infection

Autophagy displays an essential role of cellular homeostasis and also takes part in several pathologies including cancer, neurogenerative disease and infection (48, 49). N In cases of virus infection, the interaction between the autophagic machinery and the invading virus is proposed in three main outcomes: defense, avoidance and subversion (52, 53). For a cellular defense mechanism, autophagy is induced to limit the viral replication. An example of defense mechanism is supported by a study of tobacco mosaic virus (TMV). Silencing Beclin-1 or Atg6 gene in tobacco mosaic virus infected-tobacco plant leads to an increase in viral replication (17). In contrast, Herpes simplex virus types I (HSV-1) escape the fate of restriction by down regulating the pathway. It is well characterized that HSV-1 neurovirulence ICP34.5 protein, encoded by HSV-1, antagonized the function of dsRNA activated protein kinase (PKR) to suppress the induction of autophagy. Thus, HSV-1 has evolved strategies to counteract cellular antiviral function. This interaction is called avoidance (54). In addition, some viruses evolved to use autophagosomes for their replication such as poliovirus using autophagic membranes as a site for its viral replication. Inhibition of autophagy by siRNA-mediated silencing of Atg12 gene or Atg8 resulting in a reduction of viral production (17, 53).

Up to present, many Flaviviruses have been shown to subvert cellular autophagy for their benefit of viral replication (16, 54-57). Studies on Chikungunya virus (ChikV) by Krejbich-Trotot and colleagues showed induction of autophagy upon ChikV infection in HEK293. An

increase of Chikungunya virus production was seen during autophagy induction by Rapamycin, while inhibition of autophagy by using 3-MA as a pharmacological inhibitor or using RNA interference against the transcript of protein Beclin1 resulted in dramatically decreased of viral production. This study suggests that autophagy may play a promoting role in Chikungunya replication (55). For dengue virus (DENV), Panyasrivanit and colleagues showed that induction of autophagy upon DENV-2 infection in HepG2 cells is essential for viral replication (57). So, these evidences suggest subversion outcome of autophagy in ChikV and DENV. Recently, the involvement of autophagy in JEV infection was shown by Li and colleagues using human NT-2 cells. NT-2 cells were infected with JEV strain RP-9 and RP-ms. Induction of autophagy by rapamycin, biochemical autophagic inducer, increased viral production whereas down regulation of autophagy, either by using 3-methyladenine or by knockdown autophagy essential gene, Atg5 and Beclin-1, reduced viral yields suggesting a proviral role of autophagy in JEV replication (56).

In this research, JEV strains Beijing-1 and human microglial cell line were used as a model for the study of viral susceptibility and autophagy induction. Even though this viral strain is not endemic in Thailand, it induces high level of neutralizing antibodies. The induced antibodies are protective against heterologous JEV strains such that the Beijing-1 strain was used for the production of the first-generation, mouse brain-derived JE vaccine. This vaccine effectively decreases the incidence of JE in Thailand. Viral susceptibility was demonstrated by immunocytochemistry. Transmission electron microscope, indirect immunofluorescence staining and western blotting of LC3 are performed for detection of autophagy.

CHAPTER III

MATERIALS AND METHODS

3.1 Source of Materials

Chemicals

30% Acrylamide and bis-acrylamide solution	BIORAD
Ammonium persulfate	BIORAD
Calcium chloride dehydrate	SIGMA
Coomassie Brilliant Blue R-250	BIO BASIC Inc.
D-Glucose	SIGMA
DAPI (4'-6-Diamino-2-phenylindole)	Invitrogen
di-Sodium hydrogenphosphate anhydrous	Scharlau
Glycine	BIORAD
Lactalbumin hydrolysate	Fluka
Lead acetate	Electron microscopy science
Lead nitrate	Electron microscopy science
Magnesium chloride hexahydrate	SIGMA
Magnesium sulfate heptahydrate	Amersco
3-Methyladenine	SIGMA
Phosphate Buffered Saline (PBS)	SIGMA
Potassium chloride	SIGMA
Potassium dichromate	MERCK
Potassium dihydrogenphosphate	Scharlau
Seakem LE agarose	CamBrex
Sodium citrate	Electron microscopy science
Sodium Dodecyl Sulfate (SDS)	SIGMA
Sucrose	Electron microscopy science
Tetramethylenediamine(TEMED)	BIORAD

Toluidine blue	Electron microscopy science
Trichloroacetic acid (TCA)	MERCK
Tris-base	Vivantis biochemical
Triton X-100	SIGMA
Trypan Blue dye	SIGMA
Tween- 20	SIGMA
Yeast extract	MERCK

Other general chemicals and solvents used but not listed here were purchased from a variety of suppliers. All chemicals used were analytical grade.

Cell culture reagents

Dulbecco's Modified Eagle's Medium (DMEM)	HyClone
DMSO (Dimethyl sulfoxide)	SIGMA
α-modified Minimal Essential Medium (MEMα)	HyClone
Fetal Bovine Serum (FBS)	HyClone
Penicillin/Streptomycin solution	HyClone
HEPES, Free acid	HyClone
L-Glutamine	HyClone
Sodium pyruvate powder	SIGMA
0.25% Trypsin EDTA	Gibco

Cell lines

CHME-5: human embryonic fetal microglial cell line

LLC-MK2: kidney cell line from *Macaca mulatta* (Rhesus monkey)

C6/36: whole hatch larva of mosquito cell line from *Aedes albopictus*

Japanese encephalitis virus

JEV strain Beijing-1 (BJ-1) genotype III

(accession No. L48961)

Source: Human from China

Antibodies

Antibody diluents	Dako
Pan-specific anti-flavivirus monoclonal E protein antibody (A kind gift from Dr. Duncan R Smith, Mahidol University, Thailand)	
Goat anti-mouse IgG-FITC	Dako
Goat anti-rabbit IgG-horseradish-peroxidase	SIGMA
Goat anti-mouse IgG-horseradish-peroxidase	SIGMA
Rabbit polyclonal anti-MAP-LC3	SIGMA
Swine anti-rabbit IgG-TRITC	Dako

Miscellaneous

Pierce® ECL Western Blotting Substrate	Thermo fisher scientific
Pierce® BCA Protein Assay Kit	Thermo fisher scientific
Prestained Protein marker	Fermentas
PVDF (Polyvinylidene Fluoride) membrane	GE Healthcare
Shandon consul-mount	Thermo fisher scientific
Prolong Gold antifade reagent	Invitrogen

Culture media

CHME-5: Dulbecco's Modified Eagle's Medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 1%HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin

C6/36: α-modified Minimal Essential Medium (MEMα), 10% FBS, 1% L-glutamine, 1% HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin

LLC-MK2: Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, 1% HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin

3.2 Cell culture

The *Aedes albopictus* derived *C6/36* cell line was grown in α-modified Minimal Essential Medium (MEMα) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% HEPES, 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured at 28°C.

Human microglial CHME-5 and monkey kidney LLC-MK2 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplement with 10% FBS, 1% HEPES 100 units/ml of penicillin and 100 µg/ml of streptomycin. Cells were cultured in humidified atmosphere (5% CO_2/95% air) at 37°C.

3.3 Determination of cell viability

Attached cells were trypsinized and centrifuged at 1,500 rpm for 5 min at room temperature. Then, the pellet was resuspended in appropriate volume of media and pooled with the detached cells. Aliquots of 20 µl of cell suspension was mixed with 50 µl of 0.4% tryptan blue dye and 30 µl of PBS which calculated as a ratio of 1:5 dilution before counting by hemocytometer. The cells mixture was briefly vortexed and incubated for 5 min at room temperature. Aliquots of 10 µl mixture were loaded into hemocytometer for cell counting under light microscope. The live cells can be seen as unstained cells while the dead cells were identified with blue color staining. Counting cells under light microscope in four 1 x 1 mm squares of one chamber and then determined the average cell number of cells per square to calculate as the cell number/ml.

3.4 JEV propagation in C6/36 cells

The Japanese encephalitis virus (JEV) was propagated in C6/36 cells.C6/36 cells were grown in 75-cm^2 tissue culture flasks at 28°c until 80% confluence. The culture medium was discarded and washed 3 times with PBS. Subsequently, 3 ml of MEMα without FBS containing JEV strain Beijing-1 at an MOI of 1 was added. The process of viral absorption was taken for 2 hr at 28°C. The cells were supplemented with fresh culture medium and further incubated for 5 days. Aliquots of the culture medium will be stored at -80°C until use. Virus titers were determined by standard plaque assay.

3.5 Virus titration by standard plaque assay

After propagating JEV in C6/36 cell, JEV was titrated by plaque assay. The monkey kidney LLC-MK2 cells were cultured in 6-well plates for 2 days before time. Cells were washed with PBS and inoculated with 12-fold dilution of JEV in BA-1 viral diluents. Viral absorption was allowed to proceed for 120 min at 37°C with constant agitation. The JEV-infected monolayer cells were overlaid with 2x nutrient mixed with 1.6% seakem LE agarose and incubated at 37°C for 7 days. The plaques were visioned by fixing cells with the 3.7% formaldehyde for 1 hr

before crystal violet staining. The plaque formation on the monolayer of LLC-MK2 cells were counted and calculated as a JEV viral titers.

3.6 Viral infection of human microglial cells

Human microglial CHME-5 cell line was grown in 75-cm^2 tissue culture flasks. When the cells reach density of $3x10^7$cells/flask, the culture medium was discarded and washed 3 times with PBS. Subsequently, 3 ml of DMEM containing JEV at MOI 10 and 100 was added. After viruses were absorbed into the cells for 2 hr at 37°C with constant agitation, the infected cells were washed 3 times with PBS. After washing, fresh media was added and cells were incubated under standard condition.

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

CHME-5 cells were plated in 6-well plates at $7.5x10^4$ cell/well overnight. Then, the culture medium was discarded and replaced with 0.2 ml of DMEM containing JEV at MOI 10 and 100 or DMEM without FBS for mock infection. After viruses were absorbed into the cells for 2 hr at 37°C, the infected cells were washed 3 times with PBS before adding fresh media. Cells were incubated under standard condition for 5 days. Live and dead cell number determined by trypan blue dye exclusion assay were examined at 24 hr interval for 5 consecutive days using a hemocytometer.

3.8 Determination of the percentage of JEV infectivity

Approximately $1x10^5$ CHME-5 cells were cultured onto coverslips for 24 hr under standard condition. Later on, cells were either mock-infected or infected with JEV for 2 hr at an MOI of 10 and 100 and, then, incubated under standard condition for 12, 24 and 48 hr as appropriate. After that, cells were subsequently fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10 min. Cells were incubated with 5% normal horse serum for 30 min to block non-specific binding before incubating with a pan specific anti-Flavivirus antibody produced by hybridoma cell line HB-112 (58) (dilution 1:25) for 2 hr at 37°C followed with goat anti-mouse IgG-FITC secondary antibody for 1 hr at room temperature. Nuclei were counterstained with DAPI for 5 min. The coverslips were then mounted onto glass slides using Prolong Gold antifade reagent. The fluorescent signals were investigated under fluorescence microscopy (Axio observer Z-1, Carl Zeiss, Germany). For

light microscope detection, goat anti-mouse IgG-horse radish-peroxidase was used as a secondary antibody. The numbers of positive cells were counted for determining of percent infectivity (20 fields per slide).

3.9 Determination of autophagic cell death

3.9.1 Transmission Electron Microscope

Mock-infected and JEV-infected CHME-5 cells were scraped and centrifuged at 1,500 rpm for 5 min. Then, supernatant was removed. The cell pellets were immediately immersed in 3% glutaraldehyde for 1hr and post-fixed with 1% osmium tetra oxide. The pellets were dehydrated through graded series of ethanol from 50% to 100%. They were passed through two changes of propylene oxide, before being embedded in plastic media. After polymerization at 60°C for 72 hr semi-thin and ultrathin sections were cut by using ultramicrotome. The semi-thin sections (0.5 µm thick) were stained with toluidine blue, in order to select suitable sections for electron microscopy. The ultrathin sections (70-90 nm thick) were stained with uranyl acetate and lead citrate and were examined under the transmission electron microscope.

3.9.2 Western blotting

3.9.2.1 Protein extraction

The confluent 25-cm2 tissue culture flasks of mock-infected or JEV-infected CHME-5 cells were used in the preparation of protein extracts for SDS-PAGE. Before collecting the cell pellets, culture medium was discarded and cells were washed once with PBS. Then, cells were scraped in cold DMEM and pelleted by centrifugation at 1,500 rpm for 5 min. The cell pellet was lysed in RIPA buffer (50mMTris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM, 1% sodium deoxycholate, 1% SDS,1% Tritonx-100, 1X Cocktail Protease inhibitor). The cell pellets were sonicated and incubated on ice for 30 min before centrifugation at 12,000 xg for 15 min. The concentration of protein was quantified by Pierce® BCA Protein Assay Kit.

3.9.2.2 SDS-PAGE

40 µg of total protein from mock-infected or JEV-infected CHME-5 cells was separated by a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100V for 2 hr.

3.9.2.3 Western Blotting

The proteins were electrotransferred to PVDF membrane at 0.35 mA for 90 min using Mini Trans-Blot® Electrophoresis Transfer Cell. Transferred membranes were incubated with 5% skimmed milk in 0.1% TBS-tween (TBST) for 1hr at room temperature. Then, membranes were incubated with1:1000 dilution of a rabbit polyclonal anti-MAP-LC3 antibody or a 1:4000 dilution of a mouse monoclonal antibody against beta-actin in 5% skimmed milk in TBST overnight at 4 °C. Later on, the membrane was shaken for 1hr at room temperature and washing 3 times with TBST and TBS. Then, the blot was incubated with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody at a dilution of 1:10000 or HRP- conjugated rabbit anti-mouse IgG at a dilution of 1:10000 in 5% skimmed milk in TBST for 1 hr at room temperature. The signals were developed by using the Pierce® ECL Western Blotting Substrate. Band intensity volume was analyzed by Quantity One software (Bio-Rad Laboratories Inc.,).

3.9.3 Detection of autophagic marker by indirect immunofluorescence

Approximately $1x10^5$ CHME-5 cells were cultured onto glass coverslips overnight under standard condition. When the cells reached an appropriate density, the culture medium was discarded and washed 3 times with PBS. Later on, cells were either mock-infected or directly infected for 2 hr with JEV at an MOI of 100. Then, cells were washed 3 times with PBS and fresh media was subsequently added. Cells were incubated under standard condition for 48 hr. After that, cells were fixed with 4% paraformaldehyde for 20 min. Cells were washed 2 times with PBS and incubated with rabbit polyclonal anti-MAP-LC3 antibody (dilution 1:200) or a pan specific anti-Flavivirus antibody produced by hybridoma cell line HB-112 (61) (dilution 1:25) as appropriate incubate for2 hr at 37°C. Subsequently, TRITC-conjugated swine anti-rabbit IgG or FITC-conjugated goat anti-mouse IgG was further incubated for 1 hr at room temperature. Nuclei were later counterstained with DAPI. The positive numbers of punctuate LC3 staining were counted and investigated under fluorescence microscopy (20 fields per slide).

3.9.4 Chemical treatment and JEV infection in CHME-5 cells

Approximately $2x10^6$ CHME-5 cells were grown in 75-cm^2 tissue culture flasks for 24 hr under standard condition. The culture medium was discarded and replaced with complete culture medium containing 10mM 3-methyladenine (3-MA) for 3 hr before challenged with JEV at an MOI of 100 for 2 hr. Then, cells were washed once with PBS and then treated with acid glycine buffer (pH 3.0) for 1 min to wash out any un-internalized viruses. Following acid glycine treatment, cells were washed again with PBS. After washing, fresh medium was added and cells were incubated for48 hr under standard condition. The culture supernatants were collected at 24 and 48 hr. and standard plaque assay was performed for virion production.

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).

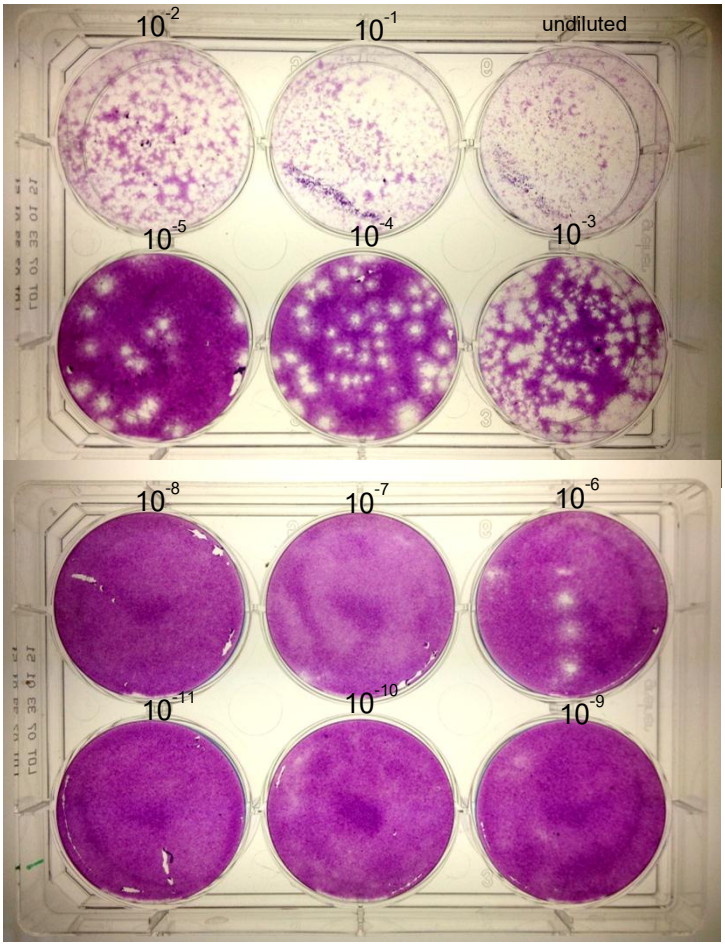


Figure 4.1 Standard plaque assay of JEV on LLC-MK-2

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.

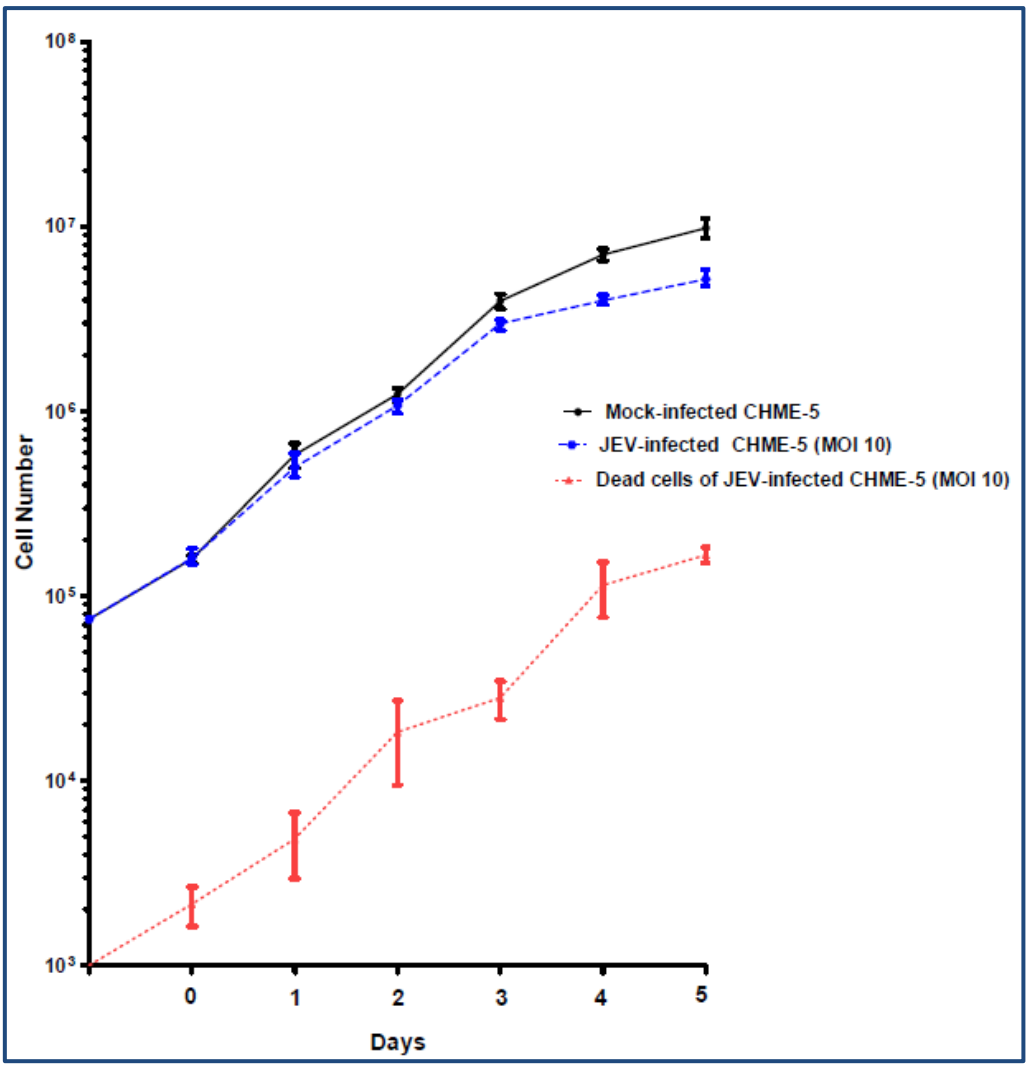


Figure 4.2 Growth curves of mock-infected and JEV-infected CHME-5 cells (MOI of 10)

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 °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 °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).

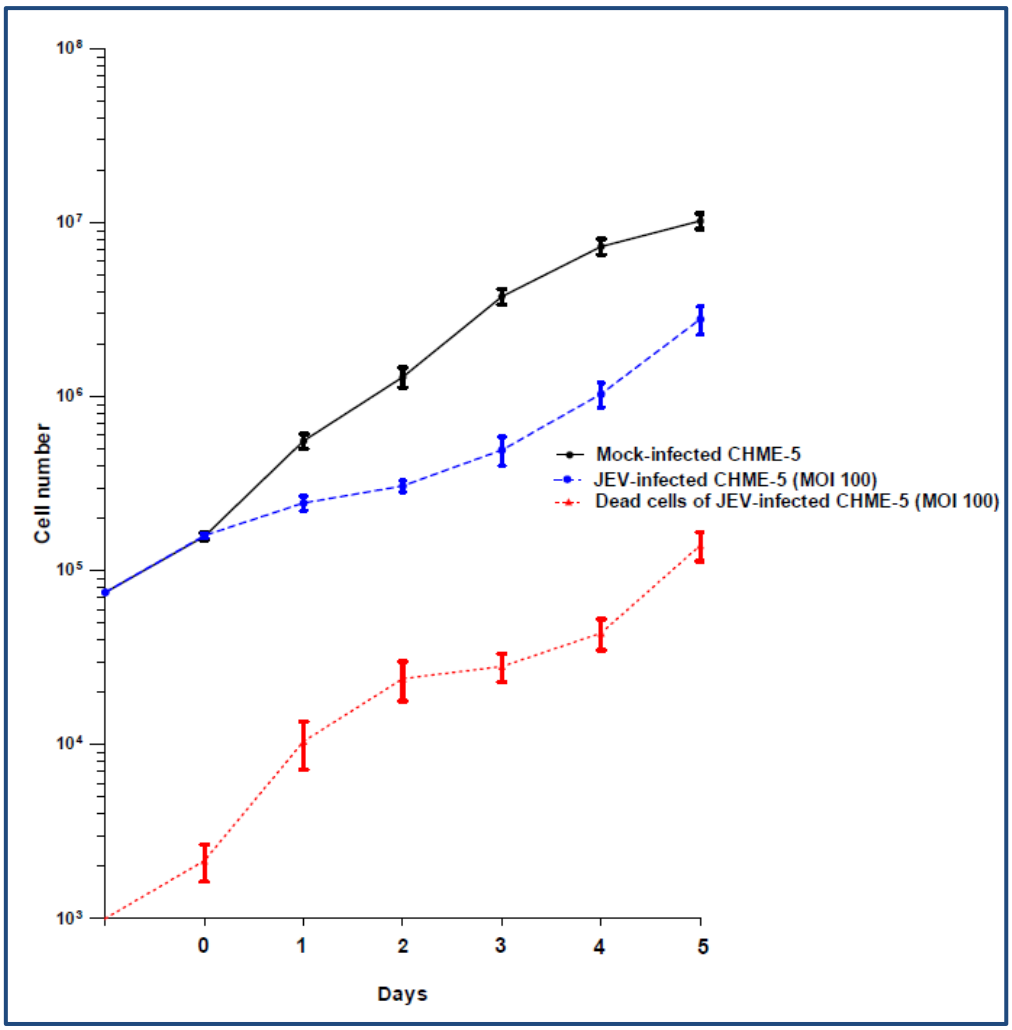


Figure 4.3 Growth curves of mock-infected and JEV-infected CHME-5cells (MOI of 100)

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.

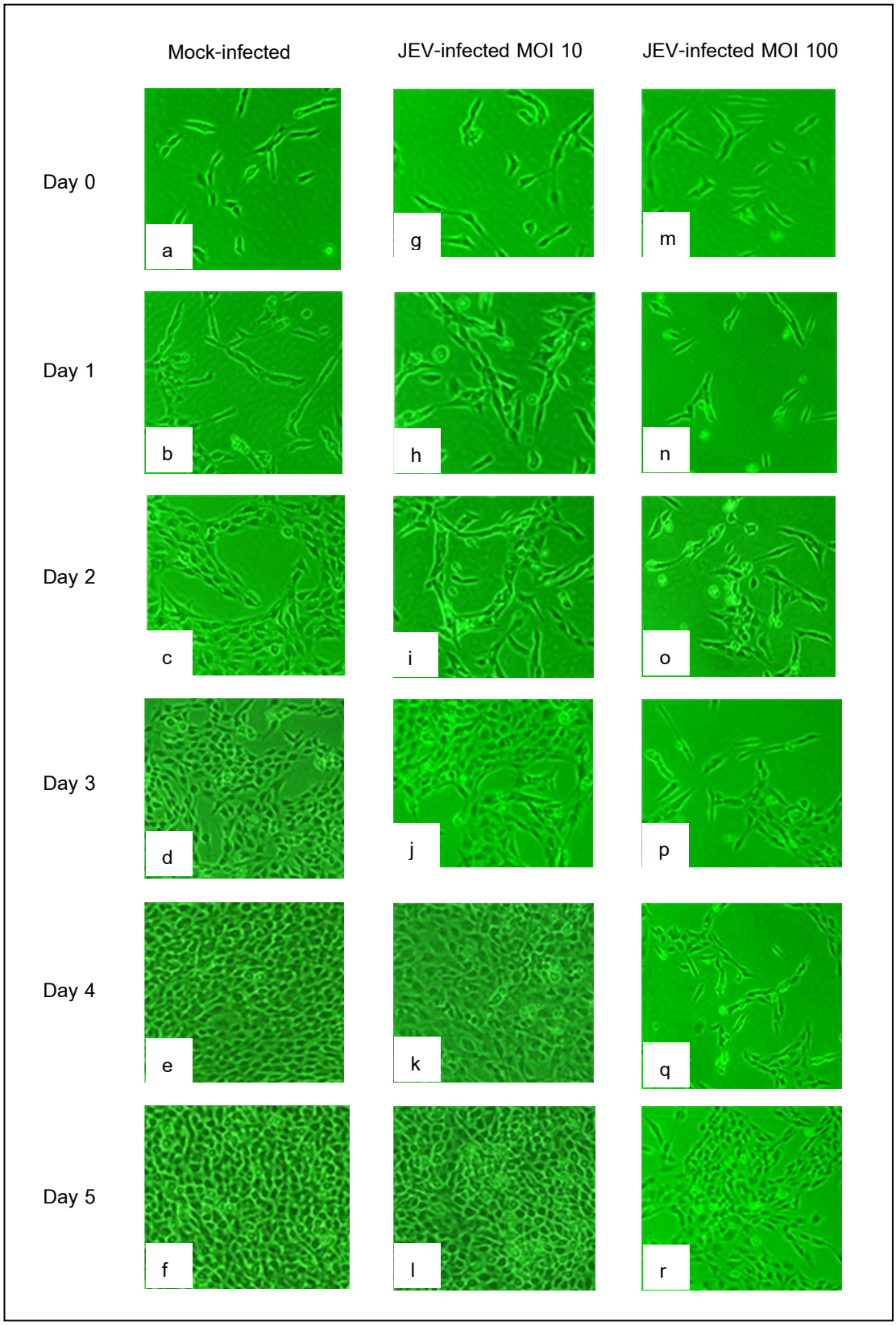

Mock-infected
JEV-infected MOI 10
JEV-infected MOI 100
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

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).

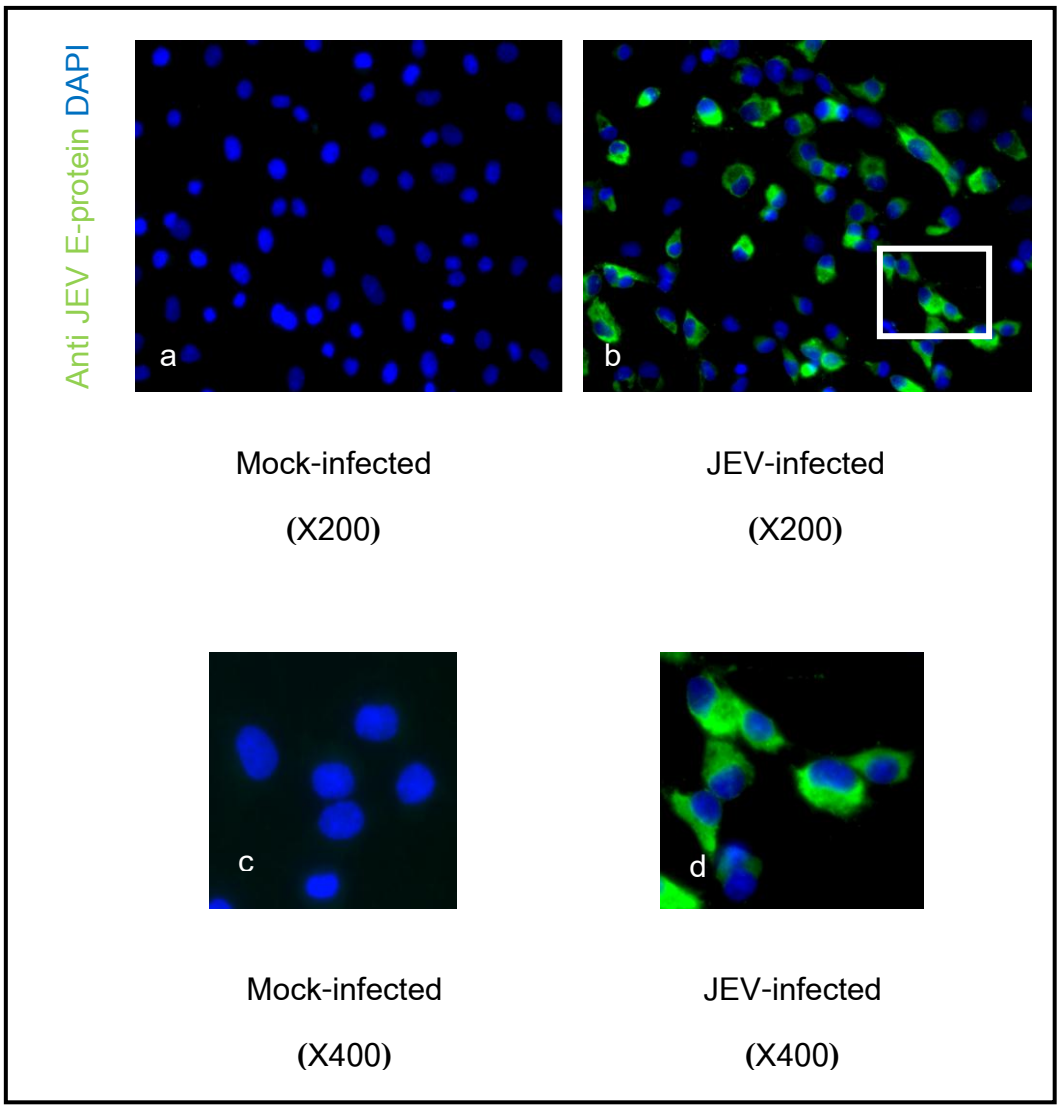


Figure 4.5 JEV infection of CHME-5 cells.

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.

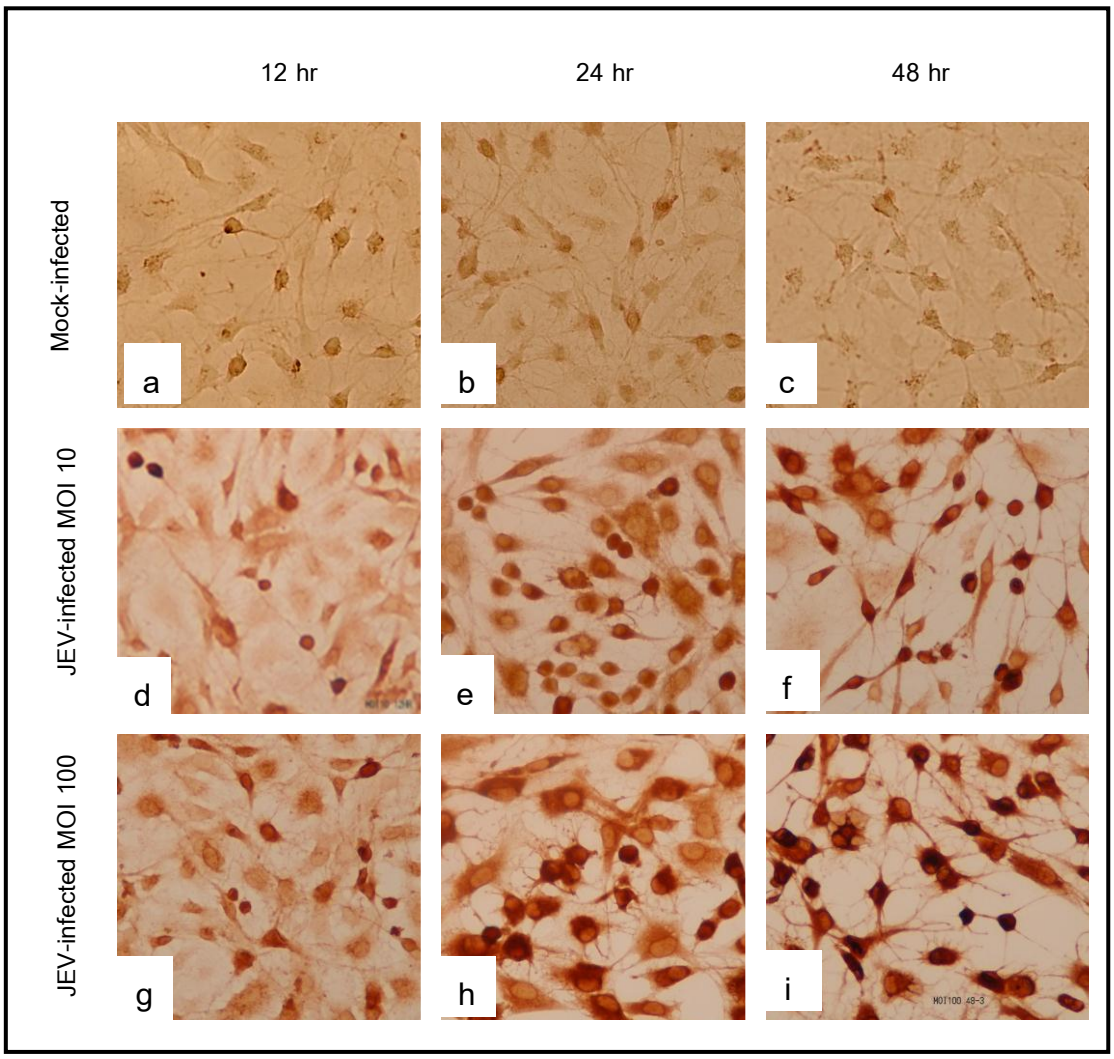


Figure 4.6 Immunocytochemistry staining of JEV-infected CHME-5 cells.

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

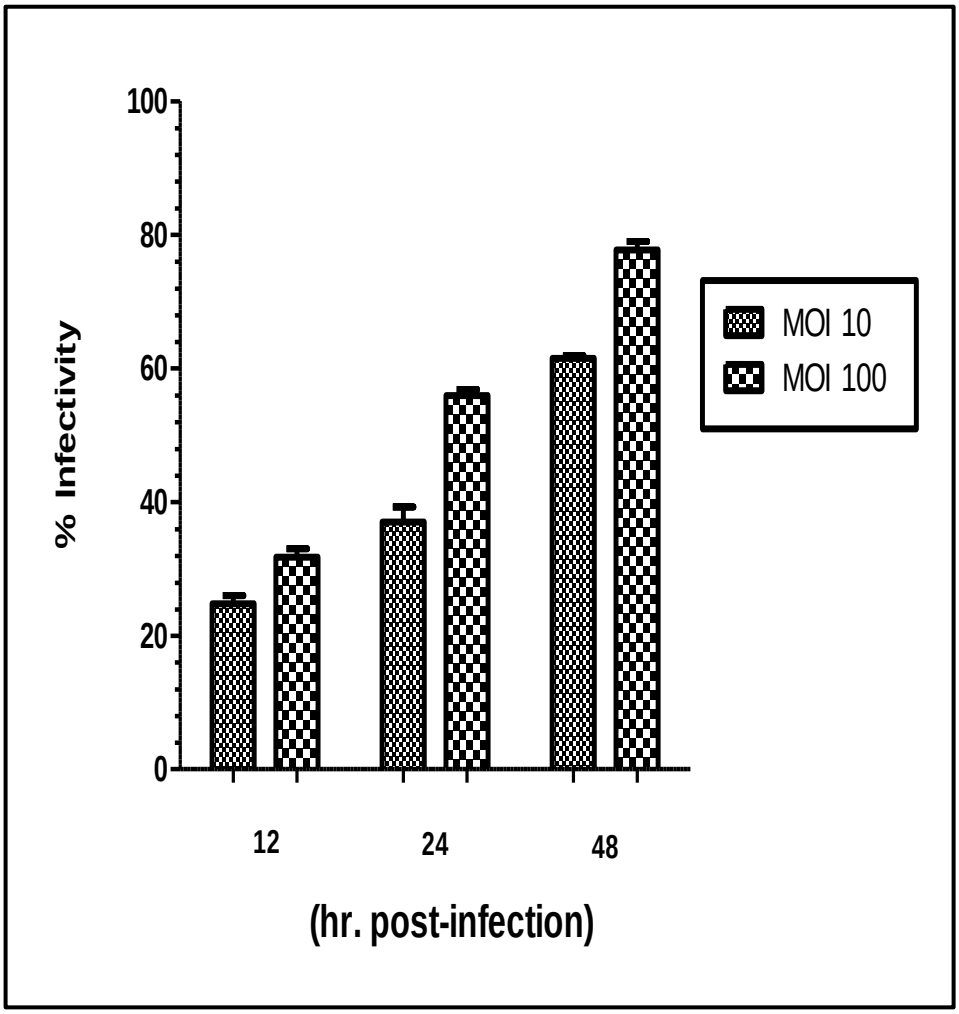


Figure 4.7 Histogram statistics of the percent infectivity determined by immunocytochemistry in JEV-infected CHME-5 cells

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).

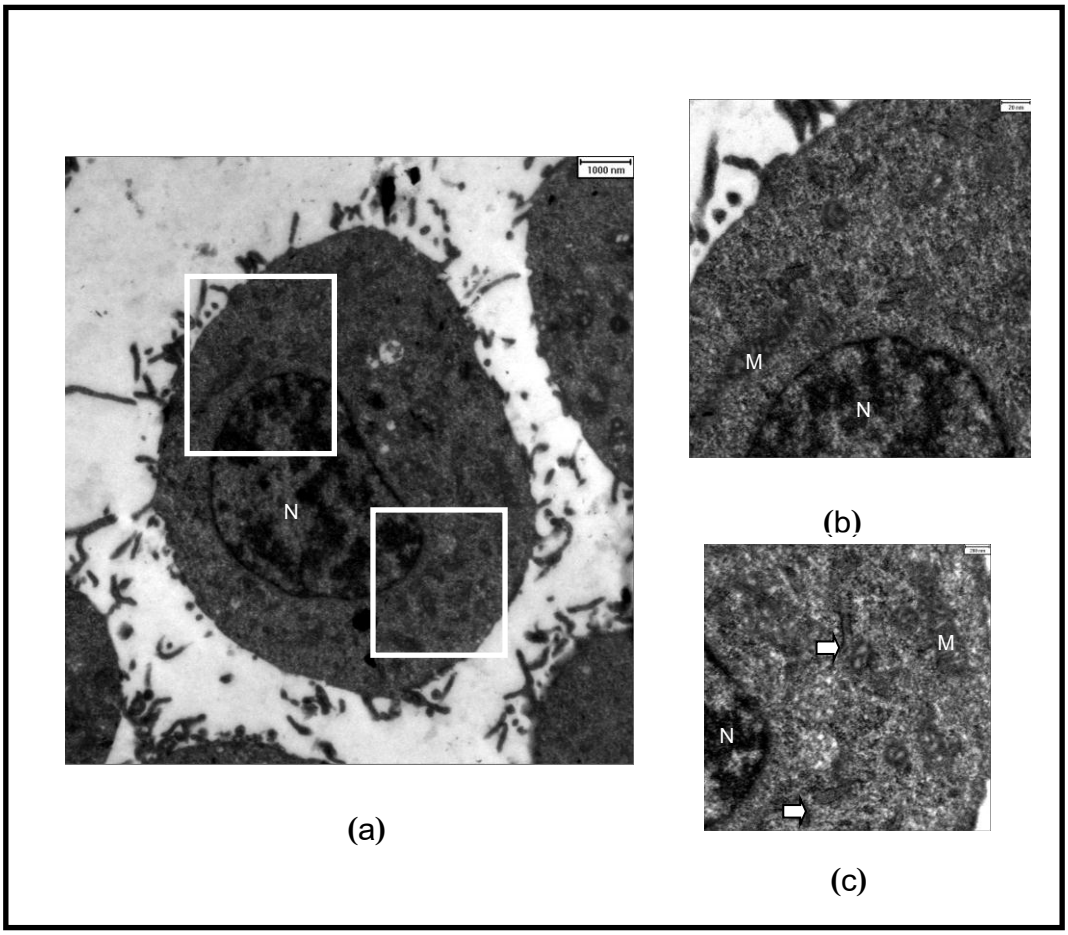


Figure 4.8 Electron micrographs of mock-infected CHME-5 cells

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

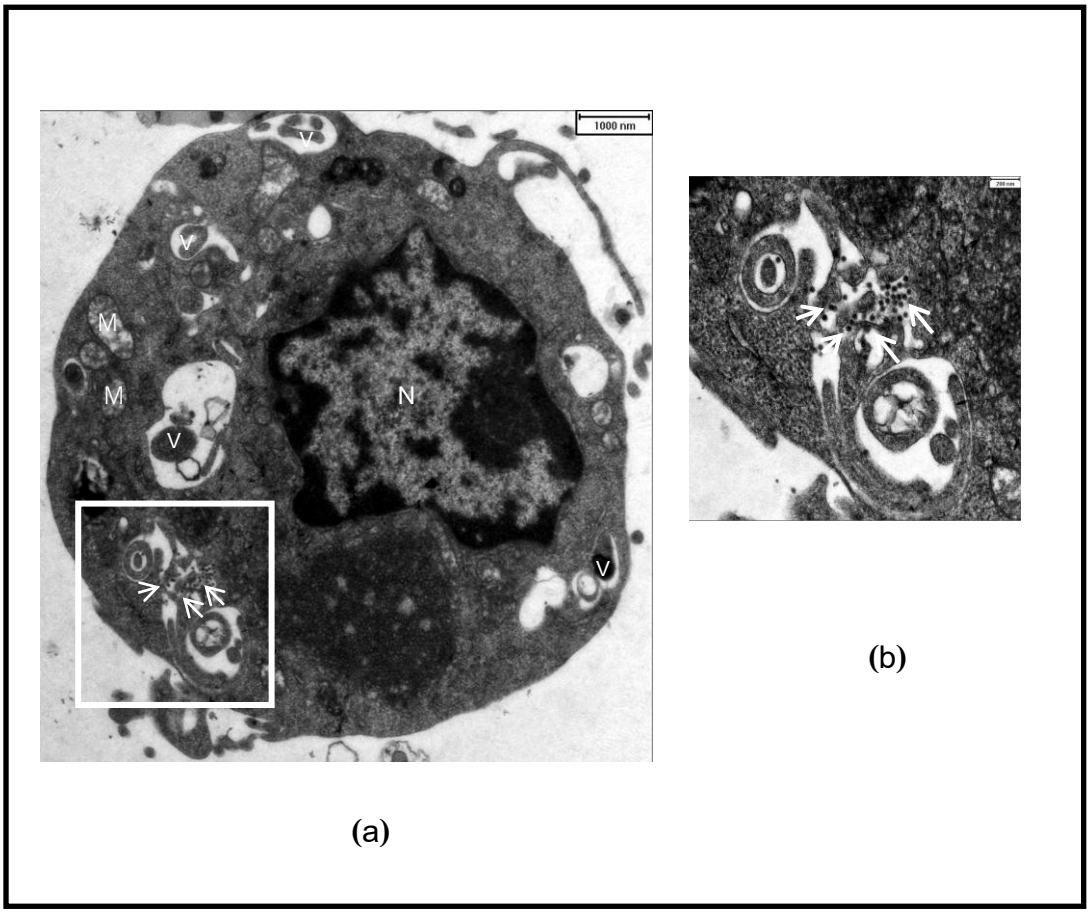


Figure 4.9 Electron micrographs of JEV-infected CHME-5 cells

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

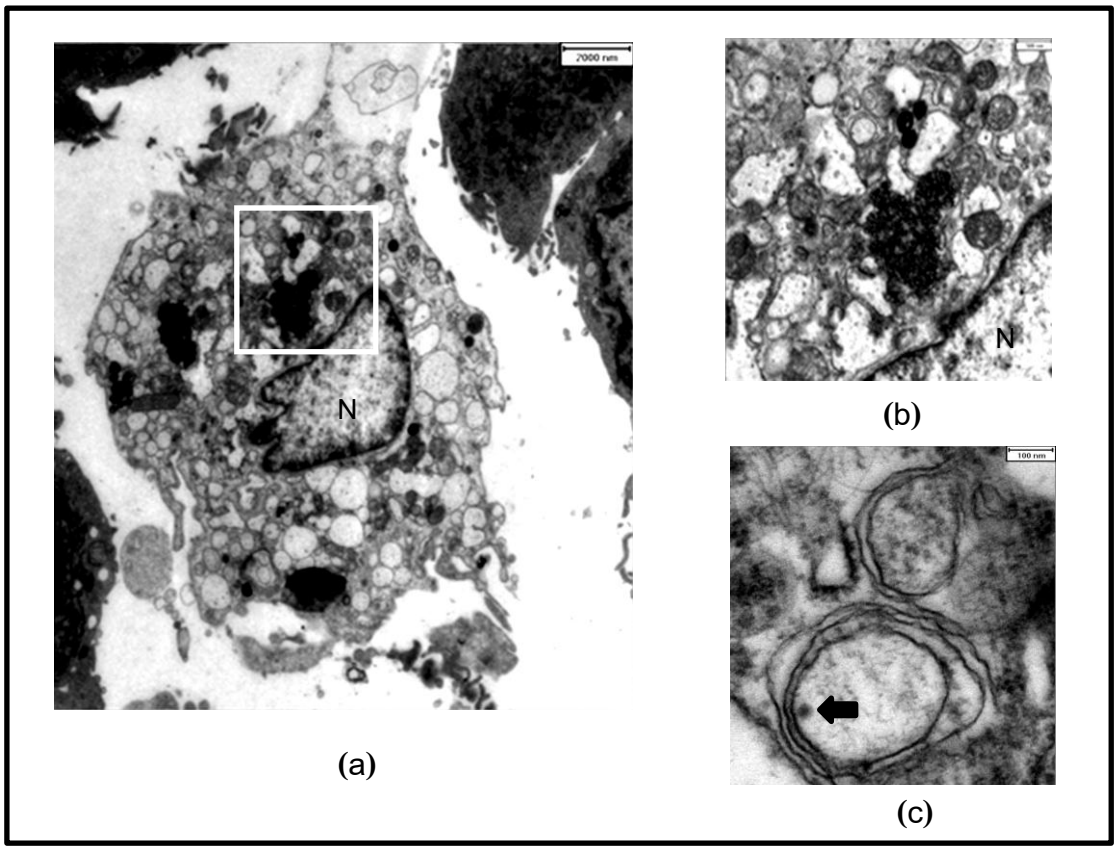


Figure 4.10 Autophagasomes in JEV-infected CHME-5 cells

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

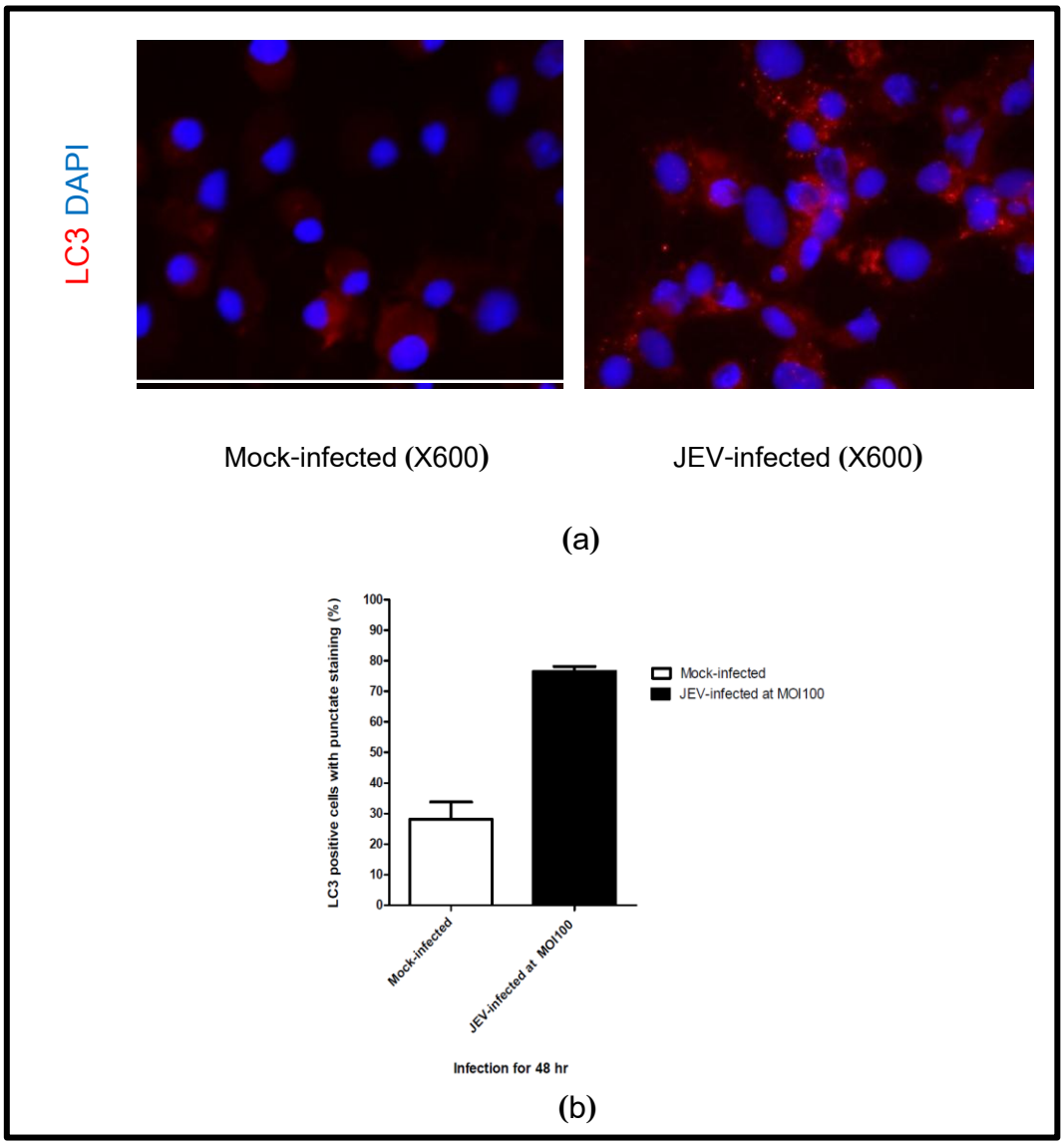


Figure 4.11 Induction of autophagy in JEV-infected CHME-5 cells

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.

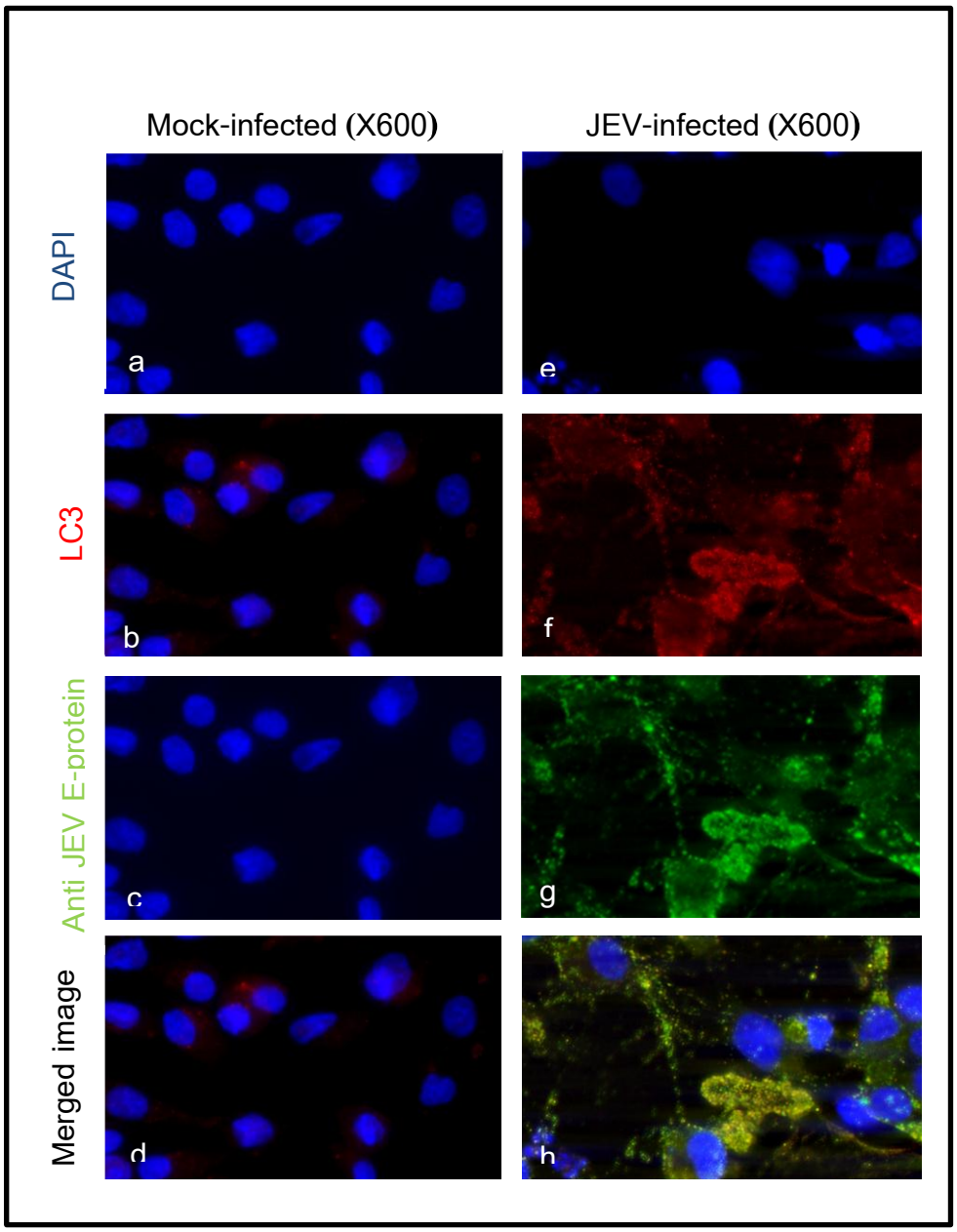


Figure 4.12 Co-localization of LC3 and JEV E-protein in JEV-infected CHME-5 cells

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.

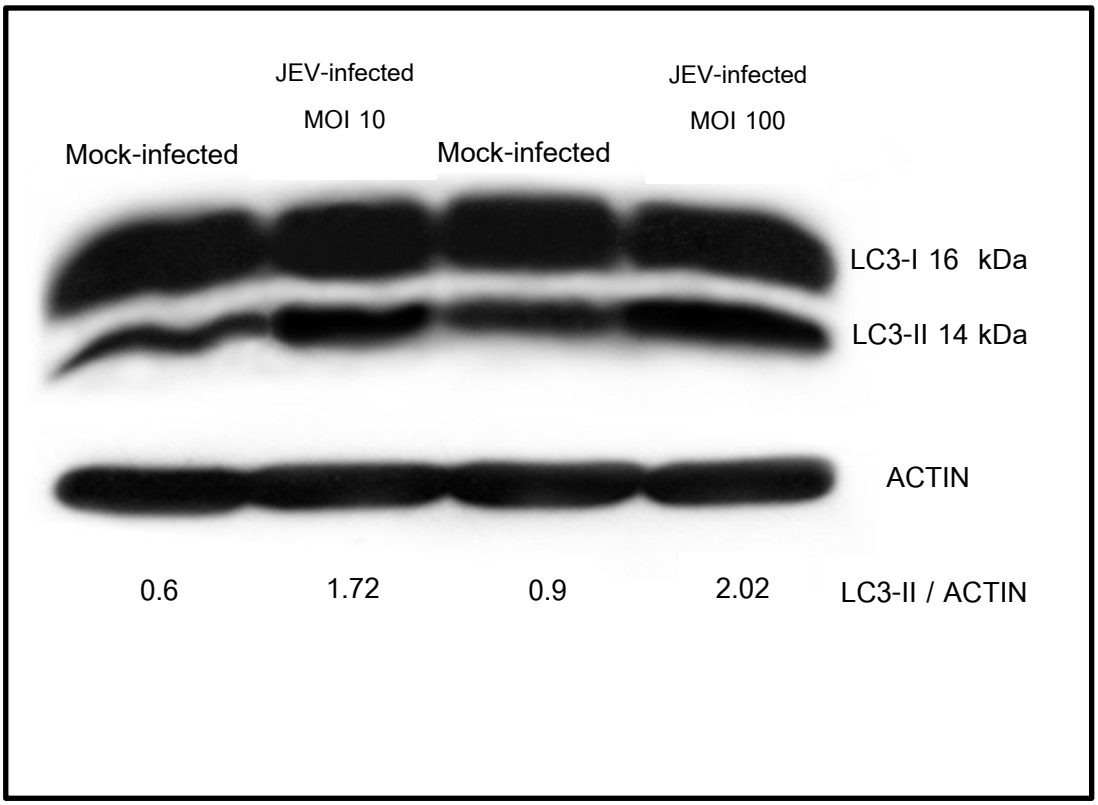


Figure 4.13 Western blotting of LC3 expression in CHME-5 cells

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.

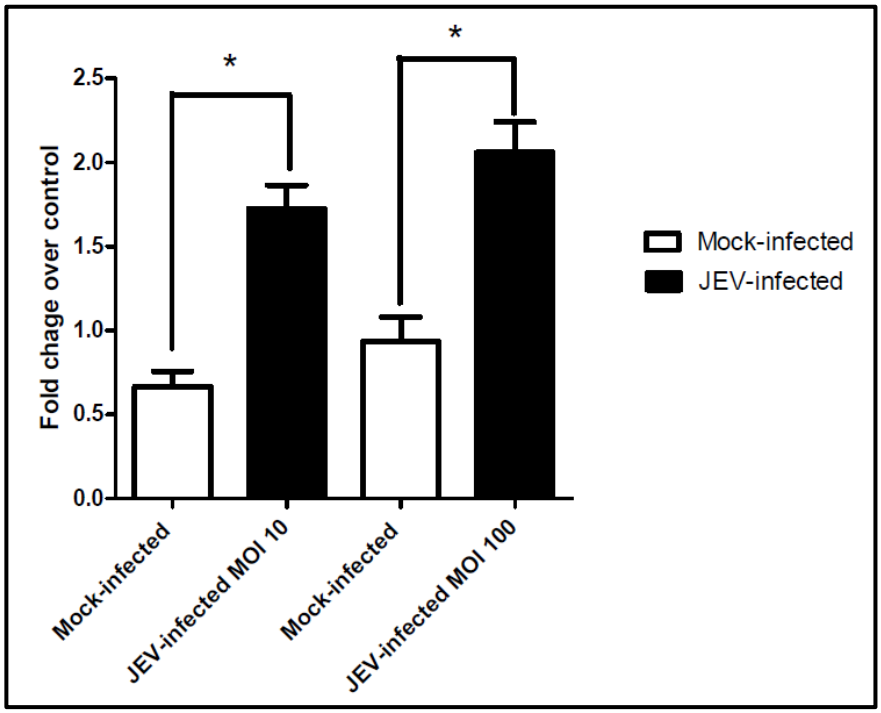


Figure 4.14 Histogram statistics of western blotting analysis of LC3-II / Actin expression in CHME-5 cells

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 (*, $p < 0.05$)

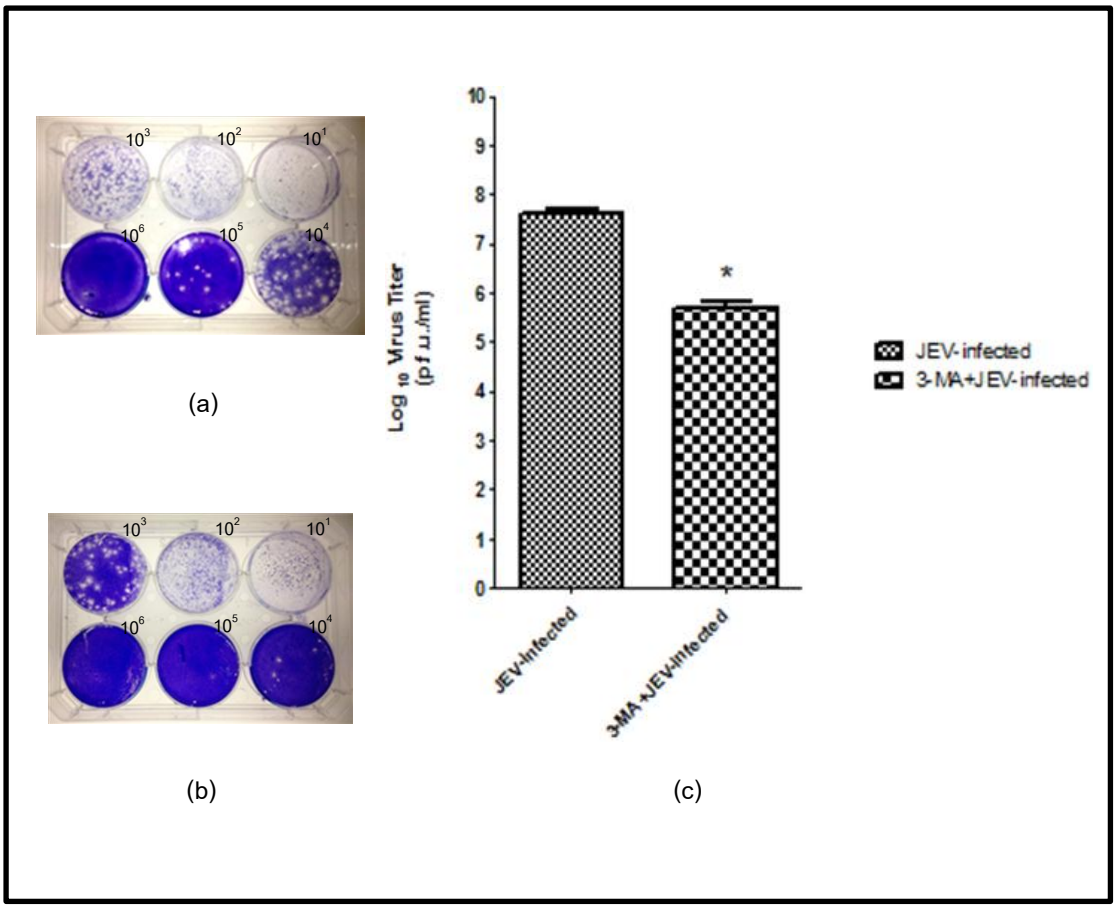


Figure 4.15 Viral production in JEV-infected CHME-5 cells in presence of autophagy inhibitor

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. (*, $p < 0.05$)

CHAPTER V
DISCUSSION AND CONCLUSIONS

Japanese encephalitis (JE) remains a public health problem in Asia (26). The clinical presentations of JE are ranging from nonspecific flu-like illness to acute fatal encephalitis (2). Upon Japanese encephalitis virus (JEV) infection, neuronal cell is a primary target of this virus in central nervous system. However, microglia, residential brain macrophages, are activated and secreted many pro-inflammatory cytokines leading to a greatly increased level of neuronal damage (10, 31, 34). Therefore, this study aimed to determine the involvement of autophagy in human microglia upon JEV infection.

Tryptan blue exclusion assay were used to determine the number of life and death cells. Infection with MOI 10 and 100 showed a few number of percent cell death (less than 10%). In addition, the proliferation rate in JEV infected CHME-5 cultures with an MOI of 100 was slower when compared to either mock-infected or JEV-infected CHME-5 cultures with an MOI of 10. This data suggested that, with a higher multiplicity of infection, a process for maintaining cellular homeostasis and survivability is triggered. The cell cycle analysis determined by flow cytometry after propidium iodine staining will provide the evidence of cell cycle arrest, resulting in a slower cell proliferation rate in response of JEV infection at last. Also, another flavivirus, Dengue virus serotype 4 (DEN-4) was reported to slow down human hematopoietic (K562) cell proliferation rate without cytopathic effects (59).

Concerning the susceptibility of human microglial cell in response to JEV infection, immunocytochemistry technique was performed. The results were quantitated as percentage of infectivity. At 48 hr post infection, approximately 60% and 78% of JEV-infected human microglial cell (CHME-5) were detected when at MOI 10 and 100 were used, respectively. Viral E protein was clearly observed within cytosol. These results showed that human microglial cells are susceptible to JEV infection, correlating to the previous study in mouse microglial (BV-2) cells (12). Interestingly, the amount of extracellular virions produced in culture supernatant of mouse microglial cells ($5x10^9$ p.f.u./ml) was approximately 2 log higher than that of human microglial cells ($4x10^7$

p.f.u./ml) at 48 hr post infection, even though mouse microglial cells were challenged with 100 times less multiplicity of infection. These imply host cell tropism and human is a dead-end host of this virus.

Autophagic cells death in human microglial cells in response to JEV infection was revealed at ultrastructural level by transmission electron microscope. In addition, immunocytochemistry and western blotting for detection of autophagic specific marker, LC3, were also performed for confirmation. These results are consistent to JEV infection in a pluripotent human testicular embyonal carcinoma cell line NT-2 cells with two different strains of viral virulence, Rp-9 and Rp-ms (attenuated) (56).

From this study, colocalization between viral E protein and LC3 was detected by using immunocytochemistry. This implies that the autophagic vacuoles may be the replication sites for JEV infection in microglial cells. To confirm that autophagosome is a replicative site for JEV , colocalization of LC3 and viral dsRNA should be performed (42).

Many Flaviviruses have been shown to subvert cellular autophagy for their benefit of viral replication. Recently research in Chikungunya virus, Dengue virus and Japanese encephalitis virus strain RP-9 and RP-ms show the induction of autophagy and suggest that autophagy may play a crucial role during flavivirus infection (16, 55-57).

Effect of autophagy on JEV viral production was studied using 3-Methylanine (3-MA) as a pharmacological autophagy inhibitor. It was found that JEV replication is dramatically decreased at 48 hr post infection. Interestingly, at 24 hr post infection, no virion was detected in supernatants of JEV-infected CHME-5 culture with 3-MA pretreatment (data not show). To further support this finding, pretreatment with autophagy inducer, Rapamycin, and genetic knockdown of autophagy essential gene Beclin-1 and Atg5 should be performed to confirm autophagy mediated JEV replication.

Recently, the reduction of viral yields was reported using genetic knockdown of Atg5 in JEV infected NT-2 cells. The level of JEV NS3 protein expression also decreased (56).

Furthermore, in 2013, Jin et al., found that the suppression of autophagy in JEV-infected neuronal cell correlated with an enhanced interferon type-1 activation. Additionally, autophagy can prolong cell survivor. This study suggests that the

autophagy negatively regulates the innate immune response to facilitate JEV infection. (60).

Understanding of details about relationship between autophagy and viral replication will improve the knowledge of JEV pathogenesis. Our finding might be applied for antiviral drug design which target to inhibit autophagosome formation resulting in preventive of viral production in host cells.

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APPENDICES

APPENDIX A

Buffers and Reagents

1. Dulbecco's Modified Eagle's medium (DMEM) stock medium	1	liter
Sodium Pyruvate powder	110	mg
Penicillin-Streptomycin solution	11	ml
HEPES, Free acid	11	ml
Store at 4°C		
2.α-modified Minimal Essential Medium (MEM-α) stock medium	1	liter
Penicillin-Streptomycin solution	11	ml
HEPES, Free acid	11	ml
Store at 4°C		
3. 1X Phosphate Buffered Saline (PBS)	1	liter
Phosphate Buffered Saline dry powder in foil pouch	1	packet
Add ddH_2O up to 1 liter and sterilize by autoclaving		
4. BA-1 viral diluent	100	ml
Filter 10X medium-199	5	ml
Autoclaved 1 M Tris-HCl pH 7.6	5	ml
2% BSA fraction V	20	ml
7.5% $NaHCO_3$	1	ml
Penicillin-Streptomycin solution	1	ml
Add ddH_2O up to 100 ml		
** Prepare freshly before use		

5. 2X Nutrient solution	100	ml
20X EBSS	9.8	ml
Ye-lah	6.6	ml
Fetal Bovine Serum (FBS)	6	ml
Gentamycin	0.5	ml
7.5% $NaHCO_3$	6	ml
Add ddH_2O up to 100 ml		
** Prepare freshly before use		

6. 1.6% Seakem LE agarose	100	ml
Seakem LE agarose	1.6	g
Add ddH_2O up to 100 ml		
Melted gel by microwave		
** Prepare freshly before use		

7. Ye-lah solution	100	ml
Yeast extracts	1	g
Lactalbumin Hydrolysate	5	g
Add ddH_2O up to 100 ml		
Sterilized by filtering through a 0.45 µm membrane filter		
Store at 4°C		

8. 2% BSA fraction V	500	ml
BSA fraction V powder	10	g
Add ddH2O up to 500 ml		
Sterilized by filtering through a 0.45 µm membrane filter		
Store at 4°C		

9. 10x TBS buffer	1	liter
Tris-HCl	121.1	g
NaCl	90	g
ddH_2O	900	ml
Adjust pH to 7.5		
Add ddH_2O up to 1 liter		
10. 1x TBS buffer	1	liter
10x TBS buffer	100	ml
Add ddH_2O up to 1 liter		
11. 10x Transfer buffer	800	ml
Tris-HCl	30.28	g
Glycine	144.13	g
Add ddH_2O up to 800 ml		
12. 1x Transfer buffer	1	liter
10x Transfer buffer	80	ml
Methanol	200	ml
Add ddH_2O up to 1 liter		
Store at 4°C		
13. 5% Skim milk in TBS buffer	50	ml
Skim milk powder	2.5	g
TBS buffer	50	ml
** Prepare freshly before use		

14. RIPA-buffer	100	ml
0.5 M Tris-HCl	10	ml
0.5 M NaCl	30	ml
0.5 M EDTA	2	ml
0.1% SDS	0.1	g
Triton X-100	1	ml
1% Sodium deoxycholate	1	g
ddH_2O	80	ml
Add dH_2O up to 100 ml		
Add 100X Protease inhibitor before use		
** Prepare freshly before use		
15. 4x Running gel buffer	200	ml
Tris-HCl	36.3	g
Adjust pH to 8.8		
Add ddH_2O to 200 ml		
Store at 4°C		
16. 4x Stacking gel buffer	50	ml
Tris-HCl	3	g
Adjust pH to 8.8		
Add ddH_2O to 50 ml		
Store at 4°C		
17. 15% Separating solution	10.07	ml
4X Running gel buffer, pH 8.8	2.5	ml
30% Acrylamide	5	ml
10% SDS	0.1	ml

10% Ammonium persulfate	65	µl
TEMED	5	µl
ddH_2O	2.4	ml
18. 4% Stacking solution	3.361	ml
4x Stacking gel buffer, pH 6.8	0.625	ml
30% Acrylamide	0.325	ml
10% SDS	25	µl
10% Ammonium persulfate	12.5	µl
TEMED	2.5	µl
ddH_2O	1.525	ml
19. 10X SDS-PAGE running buffer	1	liter
Tris-HCl	30.28	g
Glycine	144.13	g
SDS	10	g
Add ddH_2O to 1 liter		
20. 1X SDS-PAGE running buffer	1	liter
10X SDS-PAGE running buffer	100	ml
Add ddH_2O to 1 liter		
21. Coomasie blue staining	400	ml
Coomasie Brilliant Blue R250	0.4	g
Methanol	160	ml
Glacial acetic acid	40	ml
Add ddH_2O to 400 ml		

Filter through a filter paper

22. Destaining solution	1	liter
Methanol	400	ml
Glacial acetic acid	100	ml
Add ddH_2O to 1 liter		

23. 20X Earle's Balanced Salts Solution (EBSS)	500	ml
$CaCl_2 \cdot 2H_2O$	2.65	g
KCl	4	g
$MgSO_4 \cdot 7H_2O$	2	g
NaCl	68	g
$NaH_2SO_4 \cdot H_2O$	1.25	g
Glucose	10	g

Add ddH_2O to 500 ml

Sterilized by filtering through a 0.45 µm membrane filter

Store at 4°C

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