UNIVERSITI PUTRA MALAYSIA

EFFECTS OF INTRACELLULAR CALCIUM IONS IN CAUSING APOPTOSIS OF HEP-G2 LIVER CELL LINES AFTER INFECTION WITH Leptospira interrogans

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FPV 2018 28

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By

MUHAMAD SOFIE BIN MOHD HAFIZ NGOO

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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May 2018

Chair : Rozanaliza Binti Radzi, PhD
Faculty : Veterinary Medicine

Leptospirosis, is a zoonotic disease in mammals caused by pathogenic *Leptospira sp.*The most virulent species among pathogenic*Leptospira sp*. is *Leptospira interrogans.* Many features of its pathogenesis are still unknown. Calcium ions play a role as a secondary messenger in all living cells. However, level of calcium ions exceeding normal cellular threshold can cause activation of calpain leading to cell death, degradation of cytoskeletal structures and diseases progression. Cases with dogs infected with pathogenic *Leptospira sp.*, the infection is acute and most dogs die in a week after contraction. This study aims to identify the effect of intracellular calcium ions in the progression of *L. interrogans* infection onto Hep-G2 cell line.

Cell mortality was observed with Acridine orange and propidium iodine (AO/PI) stain and examined under fluorescence microscope. Cell mortality percentage were quantified with ImageJ software. Following *L. interrogans* infection on Hep-G2 cells, infected cells were withdrawn at interval period of 30 minutes, 1 hour, 3 hours and 6 hours post infection. Transmission electron microscope (TEM) and scanning electron microscope (SEM) were used to identify the infiltration of *L. interrogans*into Hep-G2 cells. Apoptosis markers (pro-caspase-3) were detected via fluorescence microscope. Intracellular calcium ions were detected using Abcam calcium ions detection kit. The expression of calpain-1 gene was assessed by using reverse transcription PCR.

During AO/PI analysis, *L. interrogans* were seen attached to cells that are clumping with high intercellular tight junctions.Mortality of Hep-G2 cells throughout the infection for multiplicity of infection (MOI) 10 (M = 16.87, SD = 9.06) was significantly lower than MOI 100. MOI 100 showed a higher degree of mortality at 30 minutes infection. SEM and TEM analysis provided evidence

of infiltration and also the formation of cell “blebbing” during Hep-G2 infection with *L. interrogans*. Pro-caspase-3 activity of Hep-G2 cells were detected during 30 minutes infection. Post infection period of 30 minutes, 1 hour, 3 hours and 6 hours shown decreased levels of calcium ions compared to control counterpart. Calpain-1 was detected at 1 hour post infection.

As a conclusion, infection of *L. interrogans*on Hep-G2caused the decrease of calcium ion in Hep-G2 cellswhich is linked to the activation of calpain-1. Calpain-1 then promotes cytoskeletal degradation (cell “blebbing”) leading to the loss of cell integrity and apoptosis of the infected host cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN ION KALSIUM INTRASELULAR DALAM MENYEBABKAN APOPTOSIS PADA HEP-G2 SEL SELEPAS DIJANGKITI DENGAN *Leptospira interrogans*

Oleh

MUHAMAD SOFIE BIN MOHD HAFIZ NGOO

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Leptospirosis, adalah penyakit zoonotik dalam mamalia yang disebabkan oleh *Leptospira sp.* patogenik. *Leptospira interrogans* adalah spesies yang paling virulan di antara *Leptospira sp* patogenik yang lain. Banyak ciri patogenesisnya masih belum diketahui. Ion kalsium memainkan peranan sebagai utusan sekunder dalam semua sel hidup. Walau bagaimanapun, tahap ion kalsium melebihi tahap selular biasa boleh menyebabkan pengaktifan calpain yang membawa kepada kematian sel, kemerosotan struktur sitoskeletal dan perkembangan penyakit. Kes-kes anjing dijangkiti *Leptospira sp*. patogenik, mengakibatkan jangkitan yang akut dengan kebanyakan anjing yang dijangkiti mati selang beberapa minggu. Kajian ini bertujuan untuk mengenal pasti kesan ion kalsium intraselular dalam perkembangan *L. interrogans* yang dijangkiti keatas sel Hep-G2.

Kematian sel diperhatikan dengan mikroskop pendarfluor dan penanda Acridine orange, propidium iodine (AO/PI). Kadar kematian sel telah diukur dengan perisian ImageJ.Setelah *L. interrogans* dijangkiti keatas sel Hep-G2, sel-sel yang dijangkiti akan dikeluarkan pada jangka masa 30 minit, 1 jam, 3 jam dan 6 jam selepas dijangkiti. *Transmission Electron Microscopy* (TEM) dan *Scanning Electron Microscopy* (SEM) digunakan untuk mengenal pasti penyusupan *L. interrogans* terhadap sel Hep-G2 dan perubahan morfologi ke sel Hep-G2. Pro-caspase-3 dikesan melalui mikroskop pendarfluor. Ion kalsium intraselular dikesan dengan kit pengesanan ion kalsium. Gen calpain-1 dikesan menggunakan *Reverse-Transcirption* PCR.

Kematian sel Hep-G2 sepanjang jangkitan untuk *Multiplication of Infection* (MOI) 10 (M = 16.87, SD = 9.06) jauh lebih rendah daripada MOI 100. MOI 100 menunjukkan tahap kematian lebih tinggi pada jangkitan selama 30 minit.

Analisis SEM dan TEM menyediakan bukti penyusupan dan juga pembentukan sel "*blebbing*" semasa jangkitan Hep-G2 dengan *L. interrogans*. . Aktiviti pro-caspase-3 sel Hep-G2 dikesan semasa jangkitan selama 30 minit. Semasa jangkitan kursus, tahap ion kalsium berkurangan secara berkala berbanding dengan kawalan. Calpain-1 dikesan pada satu jam selepas jangkitan. Semasa analisis AO/PI, *L. interrogans* dilihat menyusup ke dalam sel-sel yang berkumpul antara sel persimpangan.

Sebagai kesimpulan, jangkitan *L. interrogans* menyebabkan berkurang tahap ion kalsium yang menyebabkanpengaktifan calpain-1. Calpain-1 kemudian menggalakkan kehancuran sitoskeletal (sel "blebbing") yang mengakibatkan kehilangan sel integriti dan apoptosis pada sel.

ACKNOWLEDGEMENTS

Praise to Allah the all mighty, the most gracious and giving of glad tidings. I would like to thank my main supervisor Dr. Rozanaliza Binti Radzi for giving me the opportunity to participate as her Master degree student. She was always open for any inquiries or trouble that I would have faced during my times conducting research.I would like to acknowledge Dr. Mokrish Bin Md. Ajat for his time and positive feed backs from my research methodology, research progression until my final draft write up. I am gratefully indebted to his very valuable comments on this thesis.I would also like to acknowledge Assoc. Prof. Dr. Siti Khairani Binti Bejo for all her great remarks on bacterial culture and methodology throughout the entire study. Without her comments and suggestions, this study could have taken a turn for the worse.

I would also like to thank the experts in the Bacteriology Laboratory department from the Faculty of Veterinary Medicine, Ms Krishnammah, Ms Nur Rabiatuladawiyah, Dr. Sabri and especially En. Azri Roslan for their time and patience during which I was using their working area. Thank you again for guiding me and helping me understand the fundamental of *Leptospira sp.* culture and conducting other laboratory instruments.I would also like to express my deepest gratitude for my laboratory mates in Biochemistry Laboratory of the Faculty of Veterinary Medicine for the times we had together; during hardship and struggle, with all the valuable comments and suggestion, those were priceless moments I will remember. To Akmal, Danish and Fadzly. Also, to Afif Hizri from Mutiara Saintifik for his help in troubleshooting my work in cell culture.

Finally, I must express my very profound gratitude to my parents and my wife for providing me unfailing support and continues encouragement throughout these years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Only Allah could repay them with his surplus kindness and giving. Thank you

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LIST OF ABBREVIATIONS

^{o}C	Degree celcius
µL	Microliters
%	Percentage
µM	Micro Meter
±	Plus-Minus
Advance MEM	Advance Modified Eagles Media
AO	Acridine orange
Apaf-1	Apoptotic Protease Activating Factor 1
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
bp	Base Pair
Cm^2	Centimeter Square
CO^2	Carbon dioxide
E	Endothelial
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacitic Acid
EMJH	Ellinghausen-McCullough-Johnson-Harris
FADD	Fas-Associated Protein with Death Domain
FBS	Fetal Bovine Serum
H	Hour
IP-3	Inositol 1,4,5-Trisphosphate
L. interrogans	*Leptospira interrogans* strain Copenhageni
LPS	Lipopolysaccharide
M	Mole
mL	Milliliters
mm^3	Millimeter Cube
mm	Millimeters
mM	Milli-Mole
mg / ml	Milligram per milliliters
MOI	Multiplicity of Infection
nM	Nano-Mole
OMP	Outer membrane protein
PBS	Phosphate Buffered Saline
PI	Propidium Iodine
PTP	Permeable transition pores
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
sp.	Species
Sph	Sphingomyelinase
TAE	Tris Base, Acetic Acid and Ethylenediaminetetraacitic Acid
TEM	Transmission Electron Microscopy
VE	Vascular endothelial

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CHAPTER 1

INTRODUCTION

Leptospirosis is an epidemic disease caused by the pathogenic *Leptospira sp.*(Komi *et al.*, 2015),cases of Leptospirosis were reported in tropical climate regions such as South-East Asia, South America and the Oceania, but recent years have shown records of cases in temperate countries (e.g. France and Netherlands) (Dupouey *et al*., 2014). The major source of infection are from wild rodents; and some domestic animals that have came in contact with infected wild rodents (Benacer *et al*., 2016). *Leptospira sp.* serovar Copenhageni is the most common cause of severe human Leptospirosis with high mortality (Adler *et al*., 2011). An infection cycle of pathogenic *Leptospira sp.* starts by entering of host, which then moves on to evade the immune response and further bind to the tissues (Zhang *et al.*, 2012), they would then colonize and exit to find a new host (Hoke *et al.*, 2008). *Leptospira sp.* enters the host's body via cuts or open wound that has been in contact with infected water bodies, it can also enter via the mucous membranes route (Chirathaworn & Kongpan, 2014). They would then disseminate into the blood stream and transfer to other parts of the body, infecting organs while evading the immune system. Manifestation of infection are usually mild fever (Musso & La Scola, 2013), having similar clinical presentation as rickettsia disease, dengue and influenza; making this disease a challenge for public health staff and laboratorians to diagnose (Guerra, 2013).

Some pathogens have the ability to disperse and colonize the host's internal organs via the transmembrane proteins. Transmembrane proteins are located intercelullarly between cells and has an extremely small entry point for intercellular movement of nanoparticle between adjacent cells. Pathogenic organism have ways of exploiting the tight junctions between cells, some uses the tight junction's protein receptors as a marker for adherence and penetration, others destroy the junction as a gateway to underlining tissues, some also deploys toxin to alter the morphology of the tight junctions to inflict inflammation (Guttman & Finlay, 2009). *Treponema pallidum* and *Borrelia burgdorferi*(Barocchi *et al*., 2002) also works in the manner which utilizes the intercellular tight junctions for its dissemination into other organs (Thomas *et al*., 1988), is has been recorded that all spirocheatoses share the same spirochetemic phase during initial stage of infection (Marangoni *et al*., 2000).

Pathogens have also developed a method which influence calcium ion to be used for its propagation. *Escherichia coli* and *Vibrio cholerae* has been recorded to release toxins which influence the inositol 1,4,5-triphosphate (IP-3) to open the permeable transition pores (PTP) on the host's endoplasmic reticulum to increase the release of free calcium ion into the cytosol. The increase of calcium ion in the cytosol has been linked with the increase level of cyclic nucleotides and deregulation of chloride channels in epithelial cells, hence the increase of fluid secretion in the intestinal lumen (TranVan-Nhieu *et*

al., 2004). *Campylobacter jejuni* invasive ability into host cells has been linked with the availability of host's free calcium ions; introduction of calcium ion channel-blockers into the cell, decreases the invasion capability of the pathogen towards intestinal cells (Hu *et al.*, 2005). Calcium ion has also been linked with the replication rate of *Brucella abortus* in host macrophage (Kim *et al*., 2012).

Cell death regulated by calcium ion is very complex and proceeds in a number of ways (Fettucciari *et al.*, 2006). Despite this, an increase of calcium ion in the cell's cytoplasm could trigger apoptosis (Sharma & Rohrer, 2004). It has been recorded that only a small amount of calcium ion in the cytosol is needed to activate inositol 1,4,5-triphosphate (IP-3) in the presence of ceramide (Krishnamurthy, 1999). Ceramide are produced by breaking down of sphingomyelin in the cell membrane by various factors (Rizzuto *et al.*, 1993). It has been documented that human liver cell line are affected by the cytotoxicity of recombinant sphingomyelinase-2 (Sph-2) (Narayanavari *et al*., 2015). *Leptospira interrogans* strain Fiocruz L1-130 was shown to develop sph2 proteins when sodium chloride was added to raise the osmolarity of the culture medium (Matsunaga *et al.*, 2007).

Pathogenic *Leptospira sp.* mostly resides in the kidney, renal cavities and liver of host during infection; in addition to that, the ability to study calcium ion levels during pathogenic *Leptospira sp.* infection are ideally done on cell lines. Hence, Hep-G2 liver cell lines was chosen for this study as a liver cell model during pathogenic *Leptospira sp*. infection.

Mechanism at which leptospires utilizes calcium ions to cause host's tissue damage is still not well defined. This study examines the behavior of *Leptospira interrogans* associated with calcium ions in Hep-G2 cells. Scanning electron microscope and transmission electron microscope used in this study to determine the conditions that the cells are undergoing during infection as well as monitoring the behaviour of the bacteria throughout the whole infection process. Acridine orange and propidium iodine will be used as a viability assay, while the detection of caspace-3 activation during infection with *Leptospira interrogans* will be using the immunofluorescence method with anti-caspase-3 antibody as the primary antibody and goat anti-rabbit igG H&L as the secondary marker. Hep-G2's calpain gene expression will be identified in the gene expression assay to determine the activation of calpain by excessive calcium ion during infection.

1.1 Hypothesis of the Study

There are 2 main hypotheses to be identified in this study:

1. Cell death of Hep-G2 cells is associated with calcium ions during *Leptospirainterrogans* disease progression
2. Pathogenic *Leptospira interrogans* infection will shift the calcium ion balance in the Hep-G2 liver cells.

1.2 Aims and Objectives

The general aim is to find a link between calcium ions with the cell death of Hep-G2 cells following infection with pathogenic *Leptospira sp.* There are potential signs that calcium ion is a key player in pathogenic *Leptospira sp.* engagement with Hep-G2 cell's cell death, by understanding this mechanism, it would be an advantage to construct a concept of pathogenesis of Leptospirosis infection. Therefore, this study has laid out 3 specific objectives;

1. To determine the morphological, viability and apoptotic state of Hep-G2 liver cells infected with *Leptospira interrogans*.
2. To determine the level of calcium ion before and during infection of Hep-G2 cells infected with *Leptospira interrogans.*
3. To detect gene expression of targeted Hep-G2 genes during infection associated with calcium ion activation cascade.

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