UNIVERSITI PUTRA MALAYSIA

ELUCIDATING FELINE HEPATIC LIPIDOSIS THROUGH SERUM BIOCHEMISTRY, HISTOPATHOLOGY AND GENE EXPRESSION IN THE KLANG VALLEY STRAY CATS

MUHAMMAD FADZLY BIN SALLEH

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By

MUHAMMAD FADZLY BIN SALLEH

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

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DEDICATION

This thesis is dedicated to my father, mother, brothers and sister; to my supervisor, supervisory committee, fellow postgraduates, and friends.

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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Chairman : Mohd Mokrish bin Md Ajat, PhD
Faculty : Veterinary Medicine

Feline hepatic lipidosis (FHL) is a common metabolic dysfunction related to lipid metabolism in cats. In conditions of hepatic lipidosis, steatosis or excessive lipid deposition occurs in the liver due to the cat's inability to efficiently breakdown and utilise the excess lipids. As such, lipid vacuolation in the hepatocytes and associated clinical signs, may arise if the condition worsens. The main cause of hepatic lipidosis remains unclear. This study aims to determine the lipid composition of liver samples in Klang Valley stray cats and to relate their serum and liver biochemistry with their histopathological changes, alongside identifying expression of hepatic genes associated with energy regulation through lipid metabolism associated with FHL in stray cats.

In total, 18 stray cats that were emaciated, dehydrated and lethargic were collected from pounds located in the Klang Valley area. Alanine aminotransferase (ALT), a common biomarker to determine liver injury in small animals, was measured in cat blood serum, as well as creatinine and urea in determining kidney function. Triglycerides (TAG) and cholesterol concentrations were measured in serum through serum analysis and in liver samples through Bligh & Dyer lipid extraction to relate to their steatosis levels. Histological analysis of liver samples were carried out to determine the severity of fatty change of the liver (FCL).

Through this study, cats with increasing severity of FCL in liver histology was observed to have increased levels of serum ALT, creatinine and urea, signifying liver injury, renal challenges and dehydration. However, TAG levels in serum seemed to decrease with increasing severity of lipid vacuolation, with an increase of TAG concentration in liver sections extracted for total lipids.

Peripheral fats break down into free fatty acids and enter the liver through the blood stream, hence, the lower levels of serum TAG despite increasing severity of liver steatosis. Serum and liver cholesterol levels exhibit the same trend in TAG movement: decreasing serum cholesterol levels against increasing severity of FCL in contrast to increased liver cholesterol concentrations with FCL severity.

Expression of PPAR-γ was observed, indicating macrophage activity which may be involved in hepatic recovery as well as fatty acid oxidation. PPAR-δ expression was observed as well, a signifier of increased lipogenesis and consequently induction of inflammatory reactions.

Blood biomarkers (ALT, TAG, urea and creatinine) and liver TAG concentrations reflect on the histopathological changes in FHL cats in correlation to their FCL severity, coupled with hepatic gene expression of PPAR-δ and PPAR-γ in promoting hepatic recovery from FHL. These blood biomarkers in addition to further studies into blood-gene expression of the PPAR constituents could lead to earlier and less invasive diagnosis of FHL in cats.

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

MENJELASKAN LIPIDOSIS HEPATIK FELIN MELALUI BIOKIMIA SERUM, HISTOPATOLOGI DAN UNGKAPAN GEN PADA KUCING-KUCING TERBIAR DI LEMBAH KLANG

Oleh

MUHAMMAD FADZLY BIN SALLEH

November 2020

Pengerusi : Mohd Mokrish bin Md Ajat, PhD
Fakulti : Perubatan Veterinar

Lipidosis hepatik felin merupakan disfungsi proses metabolisme yang berkait dengan metabolisme lipid di dalam kucing. Kucing yang menghidap lipidosis hepatik akan mengalami pengumpulan lipid secara berlebihan di dalam hati disebabkan ketidakupayaan kucing mencerna secara cekap lipid-lipid tersebut serta menggunakannya. Oleh itu, pengumpulan lipid di dalam hepatosit, selain petanda-petanda klinikal yang lain bakal menjadi sekiranya penyakit ini berlarutan. Punca utama lipidosis hepatik masih lagi belum terurai. Pengajian ini bertujuan untuk menentukan kandungan lipid di dalam sampel-sampel kucing terbiar di Lembah Klang dan mengaitkannya kepada penganalisisan biokimia darah dan hati mereka, perubahan-perubahan histopatologinya, serta memastikan penyataan genetik hepatik yang berkait dengan pengawalan penggunaan tenaga melalui metabolisme lipid.

Secara kerseluruhannya, 18 kucing terbiar yang kurus kering, dinyahhidratkan, dan lesu telah dikumpul dari paun-paun di dalam kawasan Lembah Klang. *Alanine aminotransferase* (ALT), biopenanda yang lazim digunakan bagi memastikan kecedaraan hati pada haiwan-haiwan kecil, telah diukur daripada serum darah, serta kreatinina dan urea bagi memastikan kebolehfungsian buah pinggang. Paras trigliserida (TAG) dan kolesterol telah diukur di dalam serum melalui penganalisisan serum dan di dalam hati melalui pengekstrakan lipid *Bligh* dan *Dyer* bagi pengatian tahap steatosis. Analisis histologi keratan hati telah dilaksana bagi menentukan tahap keparahan perubahan lemak di dalam hati (PLH).

Pemerhatian sampel-sampel bertahap tinggi PLH pada histologinya menunjukkan jumlah ALT, kretinina dan urea menaik, menunjukkan kejadian keradangan hati, masalah renal dan penyahhidratan. Walaubagaimanapun, jumlah TAG di dalam darah didapati semakin menurun berdasarkan kenaikan keparahan PLH, tetapi terdapat kenaikan jumlah TAG di dalam keratan-keratan hati berdasarkan pengekstrakan lipid keseluruhan. Pemecahan lemak periferi menjadi asid lemak bebas akan memasuki hati melalui aliran darah, justeru, menjelaskan penurunan jumlah TAG serum dan menguatkan lagi keparahan steatosis hati. Jumlah kolesterol serum dan hati mempamerkan trend yang menyamai trend TAG: kolestrol serum semakin menurun apabila dibandingkan dengan peningkatan keparahan PLH sedangkan kolestrol hati semakin menaik selari dengan naiknya keparahan PLH.

Terdapat penyataan PPAR-γ yang menunjukkan kewujudan aktiviti makrofaj yang mungkin terlibat dalam penyembuhan hepatik selain pengoksidaan asid lemak. Penyataan PPAR-δ juga dicerap, suatu petanda ternaiknya kadar *lipogenesis* dan seterusnya penggalakan reaksi-reaksi keradangan.

Petanda-petanda darah (ALT, TAG, urea dan kreatinina) dan TAG hepatik mencerminkan perubahan-perubahan histopatologi dalam lipidosis hepatik felin yang berkait dengan keparahan PLH pada kucing, bersamaan ungkapan gen hepatik *PPAR-δ* and *PPAR-γ* yang menggalakkan kesembuhan hepatic daripada kesan PLH. Oleh itu, pengesanan awal lipidosis hepatik felin yang tak invasif mampu dilaksanakan menggunakan petanda-petanda tersebut di samping kajian lanjut pada ungkapan gen PPAR dalam darah.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Mohd Mokrish bin Md Ajat, PhD
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Goh Yong Meng, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Lau Seng Fong, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Puteri Azaziah binti Megat Abdul Rani, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 February 2022

Declaration by graduate student

I hereby confirm that:

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Name and Matric No: Muhammad Fadzly bin Salleh

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This is to confirm that:

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Signature: _____
Name of Chairman of Supervisory Committee: Dr. Mohd Mokrish bin Md Ajat

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Goh Yong Meng

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Lau Seng Fong

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Puteri Azaziah binti Megat Abdul Rani

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LIST OF ABBREVIATIONS

α-KG	α-ketoglutaric
AA	Amino acids
ADP	Adenosine diphosphate
ALT	Alanine aminotransferase
ATP	Adenosine triphosphate
2-ME	2-mercaptoethanol
CN	Carnitine
CV	Central vein
DGAT	Diacylglycerol acyltransferase
DM	Diabetes mellitus
DNL	De novo lipogenesis
ERR-α	Estrogen related receptor-α
FA	Fatty acids
FATP1	Fatty acid transport protein-1
FCL	Fatty change of liver
FOX01	Fibroblast growth factor 21
FHL	Feline hepatic lipidosis
GH	Growth hormone
GI	Gastrointestinal
GSH	Glutathione
HE	Hepatic encephalopathy
HE	Hematoxylin and Eosin
HL	Hepatic lipidosis

IBD	Inflammatory bowel disease
IL	Interleukin
LCPUFA	Long-chain polyunsaturated fatty acid
LD	Lipid droplet
LPL	Lipoprotein lipase
LPS	Lipopolysaccharides
NAC	N-acetylcysteine
NRF	Nuclear respiratory factor
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PLH	Perubahan lemak di dalam hati
PPAR	Peroxisome proliferator activated receptor
PT	Portal triad
RT	Reverse transcription
UC	Ulcerative colitis
SAMe	S-adenosylmethionine
SU	Sulphonylureas
TNF	Tumor necrosis factor
TAG	Triglycerides
TPP	Thiamine pyrophosphate
UV	Ultraviolet
VAT	Visceral adipose tissue
VLDL	Very low density lipoprotein

CHAPTER 1

INTRODUCTION

1.1 General Background of Study

The common cat, *Felis catus*, can be found in abundance in Malaysia. Many homeowners raise them for a variety of reasons including companionship, competition or even welfare. However, some domestic cats end up outside of their homes, abandoned, becoming strays on the streets. Among the common issues faced by these predatorial animals include feline hepatic lipidosis (FHL) where fatty deposition occurs in the liver of these cats. Prolonged anorexia in these stray cats force the use of all remaining fat stores, leading to the irregular build-up of the fats in the liver for storage. This study will be looking at abnormal lipid metabolism in stray cats, more specifically in FHL.

Feline hepatic lipidosis (FHL) is characterised by the excessive deposition of TAGs into the hepatocytes (greater than 80%), increasing the liver weight to more than 50% of its original weight. Secondary impairment of liver functions can occur as well as intrahepatic cholestasis (Valtolina & Favier, 2017a).

Unique dietary requirements of cats also bring concern to proper metabolic function, in particular to protein deficiency. A key feature of FHL is observed in anorectic cats where they can quickly suffer from protein malnutrition. This is due to their inability to adapt to diminishing protein resources in the body and continuously utilising the proteins, in particular, in urea cycle enzymes, and failing to conserve nitrogen (Center, 2005a). Ammonia detoxification into urea could be compromised, inflicting hyperammonemia in malnourished cats (Verbrugghe & Bakovic, 2013).

Lipidosis in cats is usually linked to anorexia, where, in healthy cats, prolonged periods of anorexia coupled with an underlying disease may lead to the development of lipidosis. This condition is coined “secondary HL”. However, healthy cats can also develop lipidosis when they undergo rapid weight loss through food deprivation, or even unintentional food deprivation from their owner, food changes that the cat may not favour, unfavourable lifestyle changes or stress. Lipidosis due to such reasons is coined “primary HL”. Evidence has shown overweight cats to exhibit FHL within two weeks in an experimental model, however, veterinarians share their experiences whereby FHL can develop faster in cases of percent losses in caloric intake against degree of adiposity, in about one week (Armstrong & Blanchard, 2009a).

In FHL, TAGs are deposited excessively in hepatocytes due to lipolysis from peripheral fat stores. The liver plays a vital role in regulating the body's energy homeostasis through metabolising glucose and fatty acids. Adenosine triphosphate (ATP), the body's main unit of energy, usually comes from the catabolism of glucose. In excess, glucose turns into glycogen for storage, which converts into fatty acids through lipogenesis. Through lipogenesis, TAGs are stored in white adipose tissue, where it can be stored virtually forever. However, in conditions of excessive food intake or impairment of fatty acid metabolism, the excessive lipids can be stored in large amounts in the liver through steatosis. In conditions where the body is low in glucose and insulin, hepatic glycogen stores are depleted and fatty acid production is reduced. Following this, adipose tissues are hydrolysed to catabolise TAGs into free fatty acids which enter the body's blood plasma. Upon reaching the liver, these free fatty acids are oxidised into ketone bodies to be used by tissues outside of the liver (Reddy & Sambasiva Rao, 2006).

1.2 Objective of Study

The welfare of stray cats in the Klang Valley is unknown, moreover if they suffer from the common metabolic disease that is FHL. Although there is much research into FHL, there is still a lack of the correlating factors between the blood biochemistry of cats with FHL, their histopathology and genetic expression related to the disease. Understanding these parameters can propose new insights into the workings of FHL.

This study was conducted to identify the biochemical parameters for cats undergoing lipidosis. The parameters of concern include blood serum and liver homogenate where several analyses were conducted respectively. Blood serum parameters reveal a broader physiological condition of the cat with liver function of concern. To go in hand with this data, liver samples were studied to identify presence and severity of lipidosis with further investigation into gene expression for lipid metabolism. Securing these data points allowed for a more wholesome investigation into lipidosis in the feline model. The study was conducted in line with these three objectives;

1. To determine serum biochemistry and lipid composition of serum and liver samples in cats in relation with FHL.
2. To assess histopathological changes associated with FHL in cats.
3. To detect targeted genes responsible for energy regulation through lipid metabolism associated with FHL from cats.

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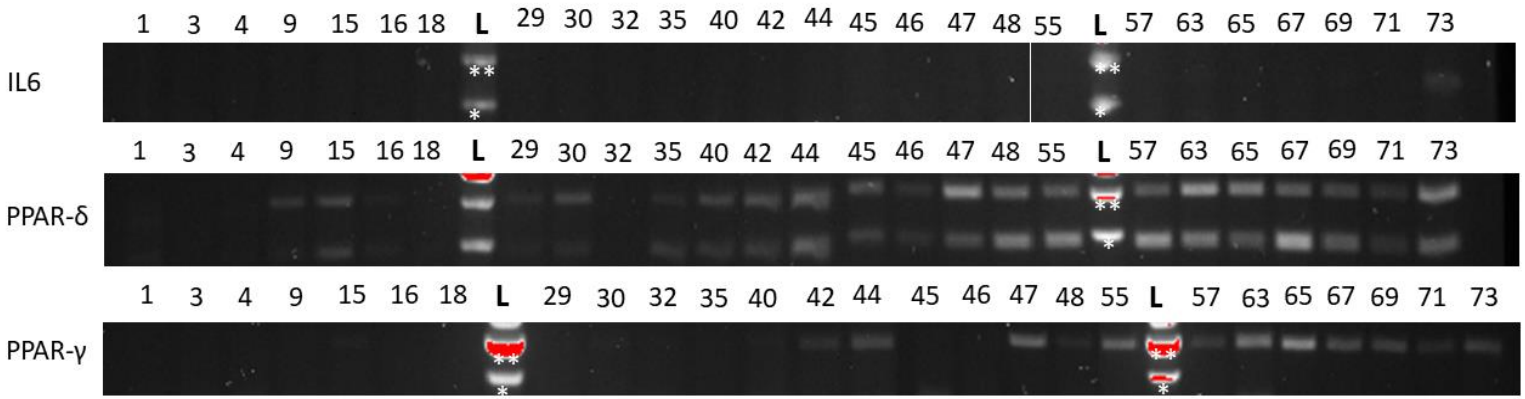
APPENDICES

Appendix A



Gene expression of Interleukin-6 (IL6). DNA ladder *300bp, **400bp. (b) Gene expression of peroxisome proliferator activated receptor-δ. DNA ladder *300bp, **400bp. (c) Gene expression of peroxisome proliferator activated receptor-γ. DNA ladder *400bp, **500bp. Numbers represent cat sample number from study

Appendix B

Sample ID	Concentration (ng/µl)	260/280 Ratio
1	444.88	2.11
3	682.16	2.11
4	505.04	2.1
9	1095.52	2.12
15	368.88	2.13
16	391.76	2.11
18	289.04	2.12
29	324.8	2.11
30	339.76	2.12
31	224.16	2.1
32	440.88	2.12
35	291.36	2.1
40	350.56	2.13
42	289.52	2.12
46	113.04	1.97
55	377.44	2.13
57	356	2.13
63	193.6	2.11
65	184.24	2.11
67	379.28	2.11
69	89.52	2.15
71	306	2.12
73	542.4	2.11

Sample ID of samples and respective concentration of extracted RNA and 260/280 ratio

Appendix C

Protocol:	Supplier
Anaesthetisation	
Item:	Virbac, Hamilton, New Zealand
Zoletil	
Euthanisation	
Item: Dolethal	Vetoquinol, Northamptonshire, United Kingdom
Blood serum separation	
Item: Eppendorf centrifuge 5702 R	Merck, Darmstadt, Germany
Liver sample storage	
Item:	
Formalin	Sigma-Aldrich, Missouri, United States of America
RNA*later* Stabilization Solution	Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania
Liver lipid extraction	
Item:	
Methanol	RCI Labscan, Bangkok, Thailand
Chloroform	
Pierce BCA Protein Assay	Thermo Fisher, Illinois, United States of America
Liver processing for histology	
Item:	
TP1020 Semi-enclosed Benchtop Tissue Processor	Leica Biosystems, Illinois, United States of America
Hematoxylin & Eosin	Veterinary Histopathology Lab, Faculty of Veterinary Medicine, UPM
Alcohol (70-100%)	
Chloroform	
Paraffin	
Xylene	
RM2045 Manual Microtome	Leica Biosystems, Illinois, United States of America
Serum biochemistry analysis	
Item:	
Automated chemistry analyer TRX 7010	Biorex, Mannheim Germany
Biolis 24i	
PCR	
Item:	
RNeasy Mini Kit	Qiagen, Maryland, United States of America
Access RT-PCR System	Promega Corporation, Wisconsin, United States of America
RNAsin™ Ribonuclease Inhibitors	
Agarose powder	
Loading dye (Blue/Orange 6×)	
DNA ladder, 25bp, 100bp	
PCR primers	Apical Scientific, Selangor, Malaysia
Sensoquest Labcycler® Thermocycler	Sensoquest GmbH, Göttingen, Germany
2-mercaptoethanol	Biorad, California, United States of America
TAE buffer	
Gel Doc XR + Image Lab Software	

List of materials used and respective suppliers.

BIODATA OF STUDENT

Muhammad Fadzly bin Salleh
fadzlyuw@gmail.com

Citizenship : Malaysian
Age : 27 (2020
Marital Status : Single

Born in 1993, Fadzly is the eldest among three younger siblings. Primary education was a mix between local studies in Selangor, Malaysia, up to Primary 3, then migrated to Wyoming, United States of America, for his 5-6th grade. His father was studying for his doctorate, allowing for the opportunity for the family to experience education in a foreign land. Upon completion of the doctorate, the family moved back to Malaysia where Fadzly continued his secondary education in Sekolah Menengah Kebangsaan Jalan Reko, Kajang, Selangor, Malaysia. There, he was introduced to the field of sciences, with teachers whom showed passion for their syllabus, sparking a greater interest in him to pursue the field in Kolej Matrikulasi Pulau Pinang, Penang, Malaysia. A year of the matriculation program allowed Fadzly to continue in the field of biology where he pursued a Bachelor's Degree in cellular and molecular biology in Universiti Putra Malaysia, Serdang, Malaysia. Challenges were faced as he delved deeper into science, culminating hard work and perseverance which allowed his graduation from the Bachelor's program with a second class honour's. Trudging on, he continued with a Master's program in the pursuit of knowledge and even greater self-discovery.

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