UNIVERSITI PUTRA MALAYSIA

LIPID SIGNALING PATHWAYS GENES EXPRESSION LEVEL IN HEPG2 CELLS SUPPLEMENTED WITH EXOGENOUS LIPID & EDIBLE BIRD NEST (EBN) EXTRACT

DARREEN A/P TAWAI

lp
FPV 2018 23

LIPID SIGNALING PATHWAYS GENES EXPRESSION LEVEL IN HEPG2 CELLS SUPPLEMENTED WITH EXOGENOUS LIPID & EDIBLE BIRD NEST (EBN) EXTRACT

DARREEN A/P TAWAI

A project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
DEGREE OF DOCTOR OF VETERINARY MEDICINE
Universiti Putra Malaysia,
Serdang, Selangor Darul Ehsan

MARCH 2018

It is hereby certified that we have read this project paper entitled "Evaluation of Lipid Signalling Pathways Genes Expression in HepG2 Cells Supplemented with Exogenous Lipid & Edible Bird Nest (EBN) Extract", by Darreen A/P Tawai and in our opinion it is satisfactory in term of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 Final Year Project.

_____

DR. MOHD MOKRISH MD. AJAT
BACHELOR OF SCIENCE (UPM), MSc (UPM), PhD (UTRECHT)
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

_____

DR. ROZAIHAN MANSOR
DVM (UPM), PhD (GLASGOW)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

_____

DR INTAN SHAMEHA ABDUL RAZAK
DVM (UPM), PhD (UPM),
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Co-Supervisor)

DEDICATIONS

To my pillars of strength, my rocks, my backbones, my muse, and my believers…It has been a rough ride, but I am here today because of all of you.

-Darreen-

If you look at what you have in life, you'll always have more. If you look at what you don't have in life, you'll never have enough.

-Oprah Winfrey-

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincerest gratitude and millions of thanks to my dedicated Biochemistry supervisor Dr. Mohd Mokrish Md. Ajat for his guidance, support and patience throughout my Final Year Project. Also to my co-supervisors, Dr Rozaihan Mansor and Dr Intan Shameha Abdul Razak for their supervision and advices.

To all the post-graduate students in Biochemistry laboratory, Mr. Fadzly, Dr. Akmal and Mr. Danish, your guidance are deeply appreciated. I am very lucky indeed to be a part of Edible Bird Nest (EBN) project and throughout the past 4 weeks, I have been exposed to various procedures and activities that I have never experienced before from cleaning the raw bird nests to culturing cells, performing PCR and running gels. I hope with these knowledge, it will be beneficial on my future endeavour.

I would also like to acknowledge my beloved parents, my pillars of strength, Mr. Tawai A/L Endin Chung, Mrs Som See A/P Ai Kelai and my siblings for their unconditional support and motivation throughout my student life. I would not have come this far without them. Last but not least, to my FYP mates, Brandon Ng Tuck Keng, Firdaus Bahrein, Firdaus Yahaya, Azzahar and Fadzillah, my FYP journey has been a fun ride with all of you.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

EBN	Edible-bird's nests
EGF	Epidermal Growth Factor
LDL	Low Density Lipoprotein
LDs	Lipid Droplets
ACAT2	Acetyl-CoA acetyltransferase 2
DGAT2	Diacylglycerol O-acyltransferase 2
LDLR	Low density lipoprotein receptor
PCSK9	Proprotein Convertase Subtilisin/kexin type 9
SREBP2	Sterol regulatory element binding transcription factor 2
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
TAGs	Triacylglycerols/Triglycerides
ER	Endoplasmic reticulum
PBS	Phosphate Buffer Saline
ATCC	American Type Culture Collection
AMEM	Advanced Modified Eagle's Medium
FBS	Fetal Bovine Serum
CC	Cholesterol Concentrate

BC	Base Control
NC	Negative Control
PC	Positive Control
TX1	Treatment 1
TX2	Treatment 2
TX3	Treatment 3
VLDL	Very Low Density Lipoprotein
PCR	Polymerase Chain Reaction
mAbs	Monoclonal antibodies

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD4999 – Projek Tahun Akhir

PENENTUAN EKSPRESI GEN-GEN YANG TERLIBAT DALAM LALUAN LIPID DI DALAM SEL HEPG2 DENGAN LIPID TAMBAHAN DAN EKTRAK SARANG BURUNG

Oleh

Darreen A/P Tawai

2018

Penyelia: Dr Mohd Mokrish Md Ajat

Penyelia bersama: Dr Rozaihan Mansor, Dr Intan Shameha Abdul Razak

"Kaviar dari Timur" ataupun lebih dikenali sebagai sarang burung walit adalah produk yang sangat bermanfaat dan berharga di mana ianya terdiri daripada rembesan air liur burung dari beberapa jenis burung walit pemakan serangga. Burung walit ini tergolong dalam famili Apodidae dan dua genera yang mempunyai tahap komersial yang paling tinggi adalah Aerodramus (burung walit bergemea) dan Collocalia (burung walit tidak bergema). Tujuan utama kajian ini dijalankan adalah untuk menentukan tahap kualitatif gen-gen yang terlibat dalam laluan lipid seperti Acetyl-CoA acetyltransferase 2 (ACAT2), Diacylglycerol O-acyltransferase 2 (DGAT2), Low

density lipoprotein receptor (LDLR), Sterol regulatory element binding transcription factor 2 (SREBP2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), Proprotein Convertase Subtilisin/kexin type 9 (PCSK9) di dalam sel mammalia HepG2 dengan ataupun tanpa lipid tambahan dan ekstrak sarang burung. Sarang burung dibersihkan, dikeringkan di dalam inkubator pada suhu 60°C dan dibiarkan semalaman. Dalam projek ini, supernatan sarang burung ditambahkan dengan aseton dengan menggunakan pada kadar nisbah 1:2 dan di simpan pada suhu -80°C selama satu jam. Sel mammalia HepG2 dikultur dan diberi rawatan mengikut Kawalan Asas, Kawalan Positif, Kawalan Negatif, Rawatan 1, Rawatan 2 dan Rawatan 3. Berdasarkan kajian ini, terdapat perubahan ketara dalam aspek kualitatif gen-gen setelah diberi ekstrak sarang burung dan lipid tambahan terutamanya terhadap gen ACAT2 dan LDLR.

Kata kunci: Sarang burung, Aerodramus spp., metabolisme lipid, ekspresi gen, PCR kualitatif

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 Final Year Project

LIPID SIGNALLING PATHWAYS GENES EXPRESSION LEVEL IN HEPG2 CELLS SUPPLEMENTED WITH EXOGENOUS LIPID AND EDIBLE BIRD NEST (EBN) EXTRACT

By

Darreen A/P Tawai

2018

Supervisor: Dr. Mohd Mokrish Md. Ajat

Co-Supervisors: Dr Rozaihan Mansor, Dr Intan Shameha Abdul Razak

"Caviar of the East" or better known as Edible-Bird Nest (EBN) is a highly valuable product composed from solidified salivary secretion of a few insectivorous swiftlet species. Swiftlets falls under the family Apodidae and the two main genera with the highest commercial values are Aerodramus (echolating swiftlets) and Collocalia (non-echolating swiftlets). The objective of this study is to determine the qualitative genes expression associated in lipid signalling pathways such as Acetyl-CoA acetyltransferase 2 (ACAT2), Diacylglycerol O-acyltransferase 2 (DGAT2), Low density lipoprotein receptor (LDLR), Sterol regulatory element binding transcription factor 2 (SREBP2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR),

Proprotein Convertase Subtilisin/kexin type 9 (PCSK9) in HepG2 mammalian cell with or without supplementation of EBN extract and exogenous lipid. Raw EBN were manually cleaned, dried in 60°C oven and left overnight. In this project, EBN supernatant were added with acetone using 1:2 ratio and kept in -80°C for an hour. Hep G2 cells were cultured in six wells plates and each wells were treated according to Base Control, Positive Control, Negative Control, Treatment 1, Treatment 2 and Treatment 3. In this study, there are significant changes in term of genes expression with supplementation of EBN extract especially in ACAT2 and LDLR genes.

Keywords: Edible bird nest, Aerodramus spp., lipid metabolism, genes expression, qualitative PCR

CHAPTER 1

INTRODUCTION

"Caviar of the East" or better known as Edible Bird Nest (EBN) is a highly valuable product composed from solidified salivary secretion of a few insectivorous swiftlet species. These swiftlets fall under the family Apodidae and the two main genera with the highest commercial values are Aerodramus (echolating swiftlets) and Collocalia (non-echolating swiftlets) (Wong, 2013). Swiftlets are distributed nearly worldwide except for coldest and treeless areas of the northen hemisphere with the highest distribution in warmer tropical regions.

According to Wong (2013), *Aerodramus fuciphagus* is the most commonly found swiftlet species in Malaysia, producing the premium grade white nest due to its composition: purely solidified salivary secretion with high concentrations of N-acetylneuraminic acid (sialic acid) and epidermal growth factor (EGF) (Looi et al., 2017). It is believed that EBN offers abundant of medicinal and health-boosting properties however there are not much scientific reports to prove this.

The objective of this study is to determine the expression level of lipid signalling pathways genes such as Acetyl-CoA Acetyltransferase (ACAT), Diacylglycerol O-acyltransferase 2 (DGAT2), Sterol regulatory element binding transcription factor 2 (SREBP2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), Low density lipoprotein receptor (LDLR), Proprotein Convertase Subtilisin/kexin type 9 (PCSK9) in HepG2 mammalian cells after supplemented with exogenous lipid and treated with or without Edible Bird Nest extract.

Hypothesis

H_o: There are no significant changes in the expression of lipid signalling genes after supplementation with exogenous lipid & EBN extract

H_A: There are significant changes in expression of lipid signalling pathway genes in the mammalian cells after supplementation with exogenous lipid & EBN extract

CHAPTER 2

LITERATURE REVIEW

2.1 Edible Bird Nest

There are 24 species of swiftlets recorded in the world which are further divided into four genera, namely Aerodramus, Collocalia (non-echolocating swiftlets, Hydrochous, Schoutedenapus but *Aerodramus fuciphagus, Aerodramus maximus*, *Hydrochous gigas, Collocalia esculent* (white belly swifts) and *Cypsiurus balasiensis* (Asian palm swift) are the top five most commonly found species in Malaysia (Lim, 2006; Ibrahim et al., 2009). According to Lim and Oswald (2004), these swiftlets breeding season usually starts from December-March followed by April-July and August-November.

Bird nests are made up of sticky secretion, mucin, which is the salivary secretion that hardened as it is built in a half-bowl shape. With the help from mucin, the nests are capable of remaining firmly attached to the walls and at the same time withstanding the weight of the eggs and hatchlings.

Salivary glands found in the swiftlet's oropharyngeal region reflect their relative capacity to produce massive amounts of salivary secretion which is important in its nest building role. Studies by Goh et al. (2001) and Marshall (1956) showed that source of this salivary secretion is believed to be from the paired sublingual glands whereby the size is influenced by the reproductive cycle. This is further confirmed based on histological findings by Shah and Aziz (2014) which reported numerous

minor salivary glands were found within the lingual apparatus and also sublingual salivary glands. The swiftlets bird nests can be completed by using the salivary secretions within 35 days period (Marcone, 2005; Kang et al., 1991) with each bird nest weighing approximately from 8-12 gram. EBN composition is mainly made up of protein (62–63%) and carbohydrates (25–27%), followed by lipids (0.14–1.28%), and ash (2.10%), though the contents of EBN are influenced by seasonal variations and breeding sites.

Some of the earlier studies have reported that EBN has various health-promoting effects and medicinal values such as pro-mitogenic effects (Ng et al., 1986), promoting epidermal growth factor (EGF)-like activity (Kong et al., 1994), pro-proliferative and immune enhancing effects (Zhang et al., 1994), anti-oxidative effects (Zhang et al., 1994), antiviral effects against influenza viruses (Guo et al., 2006), pro-proliferative and anti-inflammatory effects (Aswir et al., 2011), bone strengthening and even skin enhancing effects (Matsukawa et al., 2011).

2.2 Lipid Droplets

Lipid droplets (LDs) are the major intracellular organelles specializing in storage of neutral lipids such as triglycerides and sterol esters. The basic structure of lipid droplets comprised of the organic core made of mainly triacylglycerols (TAGs) and sterol esters with phospholipids monolayer on the outer layer (Bartz et al., 2007). LDs's lifecycle starts off with extracellular fatty acids carried by albumin and lipoproteins enter the cells or via de novo synthesize from carbohydrates.

After the formation of fatty acyl-CoA through conjugation, it is then utilized in endoplasmic reticulum (ER) to generate diacylglycerols to either converted into neutral lipids (triacylglycerols) by DGAT enzymes or enter the phospholipid synthesis pathways. Neutral lipids in LDs are mobilized by lipases to provide metabolic energy and lipids for membrane synthesis (Brasaemle, 2007; Ducharme and Bickel, 2008; Zechner et al., 2005). Excessive accumulation of lipid in LDs or disruption in energy homeostasis will result in various metabolic diseases such as obesity, diabetes, atherosclerosis and fatty liver disease (Krahmer et al., (2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Edible Bird Nest Extraction

The raw bird nests were cleaned by soaking in distilled water and manual removal of feathers and fine plumages were done by using a pair of forceps. Cleaned bird nests were dried in an oven at 60°C and left overnight. Fully dried EBN were then finely grounded by using a mortar and pestle and mixed with Phosphate Buffer Saline (PBS) in 50 ml centrifuge tube at 1:20 ratio, with each 2 gram of EBN powder added with 40 ml of PBS. To ensure the solution are thoroughly mixed, all the tubes were vortexed at speed number 5. Next, the centrifuge tubes containing EBN solution were incubated at 70°C for 5 hours.

Following 5 hours incubation, proceed with centrifugation step at 4,400 rpm at 4°C for 10 minutes. 15 ml of EBN supernatant were transferred to a 50 ml centrifuge tube, with addition of acetone using 1:2 ratio and kept in -80°C freezer for an hour. The principle behind acetone addition and freezing is to absorb/remove water from EBN, thus leaving only protein pellet at the bottom of the centrifuge tubes. After an hour in the freezer, the EBN solution was centrifuged again at 4,400 rpm at 4°C for 30 minutes and supernatant was discarded. The remaining hard protein pellet at the bottom of the tubes were slowly dissolved with PBS by using a micropipette and finally filtered with 0.22 µml filter to get a pure EBN extract.

3.2 Preparation of Complete Media

200 ml of complete media was prepared by incorporating 180 ml of Advanced Modified Eagle's Medium (1x) (AMEM) (Gibco®), 20 ml of Fetal Bovine Serum (FBS) (Gibco®), 2 ml of Penicillin-Streptomycin Mixed Solution (Stabilized) (Gibco®) (*10,000 unit/ml Penicillin; 10,000 µg/ml Streptomycin*), 2 ml of Fungizone with the formula of 90% AMEM, 10% FBS, 1% Fungizone and 1% Pen-Strep. A complete media has all its constituents and supplements added important for cell growth and proliferation. Antibiotic and fungizone were added to the media to reduce the frequency of bacterial and fungal contamination.

3.3 Cell Culture

The type of mammalian cell selected for this study is HepG2 which is a type of immortalized cell line derived from the liver tissue of a 15 year old Caucasian male with a well-differentiated hepatocellular carcinoma. HepG2 was obtained from American Type Culture Collection (ATCC) and seeded into T-75 tissue culture flask with the surface area of 7,500 mm^2. Cells were cultured in complete media comprising of Advanced Modified Eagle's Medium (1x) (AMEM) supplemented with 10% of Fetal Bovine Serum (FBS), 1% of Penicillin-Streptomycin Mixed Solution (Stabilized) and 1% Fungizone for 2 days in a 37°C incubator with 5% CO_2. The cells count at 100% confluency in a T-75 culture flask is 8.4 X 10^6.

3.4 Cells Treatments

The old media in T-75 flask was removed by using a 10 ml seropipette and washed twice with PBS to remove the old media on the cell surface. Cells were then harvested from T-75 tissue culture flask via dissociation in 0.25% Trypsin and incubated for 3 minutes in an incubator at 37°C to allow the cells to dissociate and detach from the flask surface.

Complete media was added to inactivate Trypsin activity and the solution was transferred into a 15 ml centrifuge tube for centrifugation at 1,300 rpm for 3 minutes. Supernatant was discarded and complete media was added in the centrifuge tube to a final volume of 10 ml. The media was suspended a few times to fully dissociate the cell clumps at the bottom of the tube.

For cell count procedure, 10 µl of the solution was thoroughly mixed with 10 µl of Trypan Blue in a 1 ml minicentrifuge tube. Next, 10 µl of the mixed solution was pipetted on each side of the haemocytometer for cell quantification observed under inverted microscope. Following rough cell count estimation by using haemocytometer slide method, cells were seeded into triplicates of 6 well plates with complete media and incubated at 37°C for 2 days. Cells confluency were approximately 70-80% at the point of cells treatments.

Each wells were treated according to its respective treatments with Base Control: 99% AMEM and 1% FBS (Complete media), Negative Control: Complete media with 250X Cholesterol Lipid Concentrate and 2.5mg/ml Low-Density Lipoprotein from human plasma, Positive Control: Negative Control with addition of Simvastatin, Treatment 1: Negative Control with supplementation of 0.5 mg/ml EBN, Treatment 2: Negative Control with supplementation of 1.0 mg/ml EBN and Treatment 3: Negative Control with supplementation of 1.5 mg/ml EBN.

Treatment	AMEM	FBS	LDL	CC	Simvastatin	EBN
BC	1980 µl	20 µl	-	-	-	-
NC	1980 µl	20 µl	8 µl	16 µl	-	-
PC	1980 µl	20 µl	8 µl	16 µl	8 µl	-
TX1	1980 µl	20 µl	8 µl	16 µl	-	426 µl
TX2	1980 µl	20 µl	8 µl	16 µl	-	853 µl
TX3	1980 µl	20 µl	8 µl	16 µl	-	1279 µl

Table 1: *Media compositions of different cell treatments*

3.5 RNA Extraction and cDNA Synthesis

After 24 hours of cells treatments, cells were harvested through trypsinization with 0.5 ml pre-warmed Trypsin in each well and incubated at 37°C for 3 minutes to allow for cells dissociation and detachment. Next, 0.5 ml of complete media was added in to inactivate Trypsin activity. Using a 2 ml seropipette, the solution was suspended a few times and transferred into a 15 ml centrifuge tube. Another 1 ml of complete media added into the well and swirled around to harvest the remaining cells in the well. Following cell harvesting step, the solution was centrifuged at 1,300 rpm for 3 minutes and supernatant was removed leaving only the cell pellet. Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The integrity and purity of the extracted RNA were checked using TECAN infinite M200 PRO and samples were kept at -20°C freezer until further analysis can be made.

3.6 Conventional PCR Amplification

Conventional PCR was performed using Access RT-PCR System Mix by Promega and primers purchased from Integrated DNA Technologies. The PCR mixture included 31.5 µl Nuclease –free water, 10 µl AMV/Tfl 5X Reaction Buffer, 0.5 µl dNTP Mix (10 mM), 2 µl $MgSO_4$ (25 mM), 1 µl AMV Reverse Transcriptase (5µ/µl), 1 µl Tfl DNA Polymerase (5µ/µl), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM) and 2 µl RNA template (10 pg-1 µg) with each reaction had a final volume of 50 µl. Primers were diluted by mixing 10 µl to 90 µl of Nuclease-free water.

For the analysis, 2 µl of total RNA was used from each treatment. PCR amplifications were performed on a thermal cycler (Labcycler by SensoQuest) with 40 cycles at 94°C, 30 s; 55°C, 1 min; 72°C, 1 min for **ACAT2** gene; 32 cycles at 95°C, 15 s; 55.4°C, 15 s; 72°C, 16 s for **DGAT2**; 45 cycles at 95°C, 1 min; 64°C, 1 min; 72°C, 20 s for **LDLR** gene; 45 cycles at 94°C, 30 s; 60°C, 1 min; 68°C, 2 min for **SREBP2**; 30 cycles at 95°C, 1 min; 63°C, 1 min; 72°C, 20 s at **PCSK9**; and 40 cycles at 94°C, 30 s; 55°C, 1 min; 72°C, 1 min for **HMGCR** gene. Amplification of PCR products were confirmed using 1.5% and 2% agarose gel electrophoresis, depending on the base pair size of the genes tested.

Primer sequences used for PCR were as follow (forward; reverse): **ACAT2** (5'-GCC TCA GAC AAT ACA ATG G-3', 5'-AAA CAC GTA ACG ACA AGT CC-3'), **DGAT2** (5'-AGT GGC AAT GCT ATC ATC AT, 5'-GAG GCC TCG ACC ATG GAA GAT-3'), **LDLR** (5'-CCC CGC AGA TCA ACC CCC ACT C-3', 5'-AGA CCC CCA GGC AAA GGA AGA CGA-3'), **HMGCR** (5'-GGG ACC AAC CTA CTA CCT C-3', 5'-GTC GAA GAT CAA TTT ACA A-3'), **SREBP2** (5'-AAC GGT CAT TCA CCA GGT C-3', 5'-GGC TGA AGA ATA GGA GTT GCC-3') and **PCSK9** (5'-TCC ACG CTT CCT GCT GC-3', 5'-CAC GGT CAC CTG CTC CTG-3')

3.7 Gel electrophoresis

1.5%* and 2%** agarose gels were prepared by mixing 3.75 gram of agarose powder in 250 ml of 1X TAE Buffer and 5 gram of agarose powder in 250 ml 1X TAE Buffer respectively in the conical flask. 1 µL of Invitrogen SYBR Safe DNA gel stain was added for every 10 ml of agarose solution. Stained agarose solution was carefully mixed to ensure no bubble formation. The gel was gently poured into the gel casting apparatus and wait to solidify for approximately 20-30 minutes. Once the gel solidified, the comb was gently taken out from the solidified agarose gel and the gel was transferred into BIO-RAD electrophoresis device and immersed with 1X TAE Buffer.

2 µl of 100bp DNA ladder (Invitrogen) was mixed with 2 µl of loading dye (Invitrogen) on the parafilm and pipetted in the marker lane which serves as a set of standards which is used to compare with the size of the samples on the gel. Next, 4 µl of amplified RNA from each samples was mixed thoroughly with 2 µl of loading dye on the parafilm and loaded into the dedicated well on the gel. The voltage was adjusted to 100 Volt for 30-40 minutes. Stop the migration once the front dye has reached the second last lane of the gel. Finally, place the gel in Molecular Imager® Gel Doc™ XR+ Imaging System with Image Lab™ Software to read the expression of genes on the agarose gel.

**1.5% Agarose gels were used for ACAT2, DGAT2, LDLR, HMGCR genes*

***2% Agarose gels were selected for PCSK9 and SREBP2 genes*

CHAPTER 4

RESULT

4.1 Acetyl-CoA acetyltransferase 2 (ACAT2)

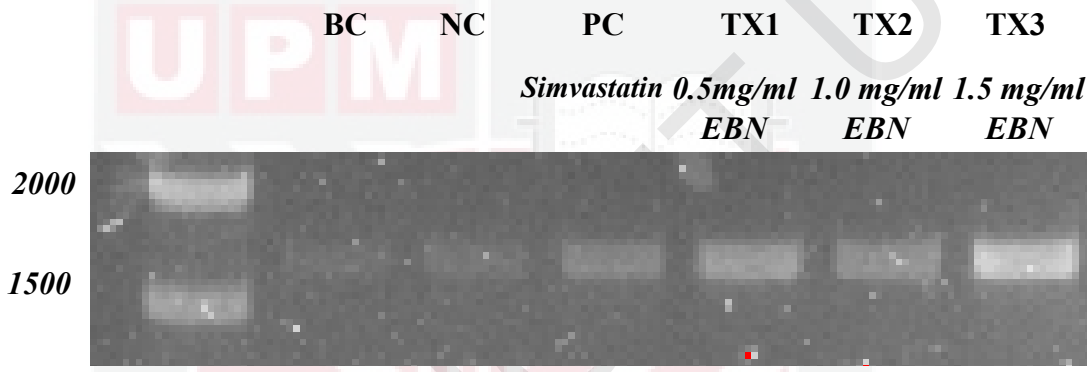

Figure 1: Analysis of ACAT2 gene expression in HepG2 cells using RT-PCR. Monolayer culture of HepG2 cells were grown as described under "Cell Culture". Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The PCR product predicted to be 1665 bp, were resolved on SYBR Safe (Invitrogen) stained 1.5% agarose gel alongside a 1kb DNA ladder (Promega).

The function of ACAT2 is to convert sterol to sterol ester for the synthesis of Very Low Density Lipoprotein (VLDL) and cytoplasmic lipid droplets. In comparison to Base Control at which the HepG2 cells were only supplemented with complete media (AMEM) and Bovine Calf Serum (BCS), the cells treated with EBN extract have shown to have significant upregulation of ACAT2 gene expression. The upregulation of the gene increases with the concentration of EBN extract supplementation and this

proves that EBN extract gives a more desirable result as compared to the Positive Control with Simvastatin supplementation.

4.2 Diacylglycerol O-acyltransferase 2 (DGAT2)

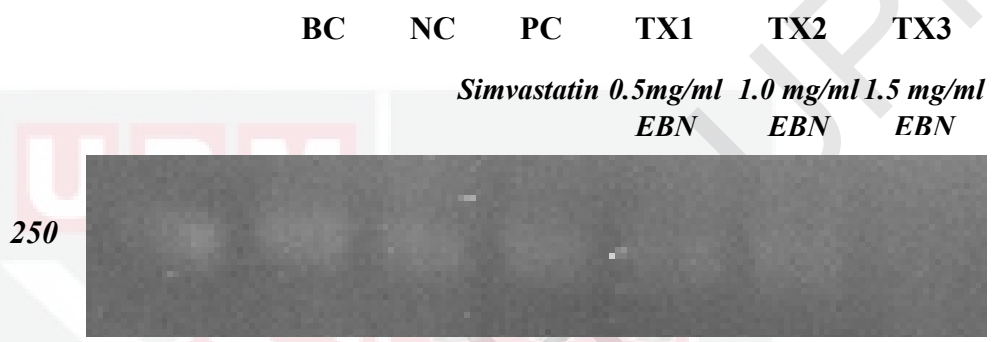

Figure 2: Analysis of DGAT2 gene expression in HepG2 cells using RT-PCR. Monolayer culture of HepG2 cells were grown as described under "Cell Culture". Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The PCR products predicted to be 270 bp, were resolved on SYBR Safe (Invitrogen) stained 1.5% agarose gel alongside a 1kb DNA ladder (Promega).

DGAT2 catalyses triacylglycerol (TAG) synthesis using diacylglycerol & fatty acyl CoA as substrates. Based from the results shown above, there are not much signifcant differences in term of DGAT2 expression in Treatment 1, 2 and 3 in comparison with the base control, negative control and positive control. In short, there are no significant differences in term of the level of DGAT2 gene expression with EBN extract supplementation and exogenous lipid concentrate in HepG2 mammalian cells.

4.3 Low Density Lipoprotein Receptor (LDLR)

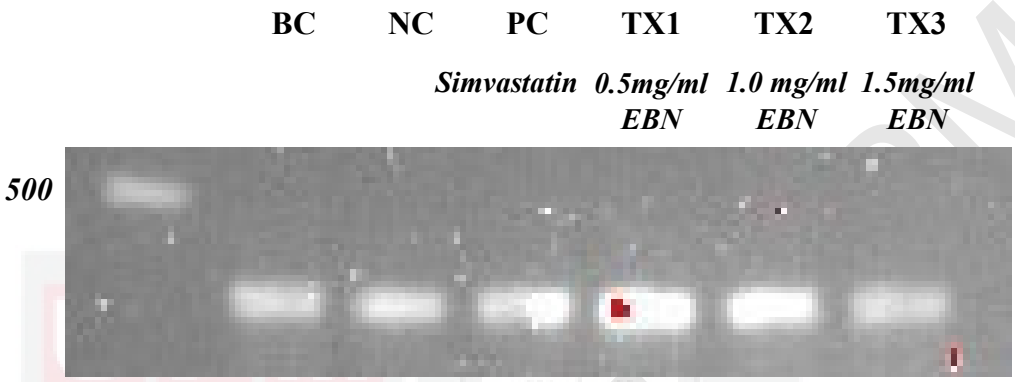

Figure 3: Analysis of LDLR gene expression in HepG2 cells using RT-PCR. Monolayer culture of HepG2 cells were grown as described under "Cell Culture". Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The PCR products predicted to be 369 bp, were resolved on SYBR Safe (Invitrogen) stained 1.5% agarose gel alongside a 1kb DNA ladder (Promega).

This gene is responsible for the regulation of LDL receptor located on the outer surface of the cells where the receptors will bind to LDLs and transport them into the cell. With supplementation of EBN extract, the LDLR gene is upregulated and the effect is much more significant compared to Simvastatin drug. However, it seems as if the LDLR expression is downregulated as the EBN concentration increases.

4.4 Sterol Regulatory Element Binding Transcription Factor 2 (SREBP2)

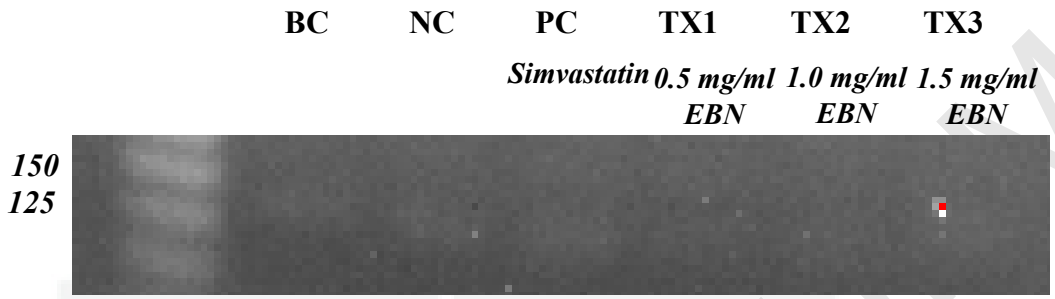

Figure 4: Analysis of SREBP2 gene expression in HepG2 cells using RT-PCR. Monolayer culture of HepG2 cells were grown as described under "Cell Culture". Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The PCR products predicted to be 133 bp, were resolved on SYBR Safe (Invitrogen) stained 2.0% agarose gel alongside a 25bp DNA ladder (Promega).

SREBP2 is activated when the intracellular sterol level in the endoplasmic reticulum (ER) is low. The targeted genes such as HMGCR, LDLR and PCSK9 are related to cholesterol biosynthesis and uptake. Based from the result shown above, there are no significant differences in term of SREBP2 gene expression with supplementation of EBN extract and exogenous lipid concentrate in HepG2 mammalian cells. Treatment 1, 2 and 3 showed a merely same level of SREBP2 expression regardless of EBN concentration which could suggest that different concentration of EBN supplementation do not show any significant differences for SREBP2 gene expression level.

4.5 Proprotein Convertase Subtilisin/kexin Type 9 (PCSK9)

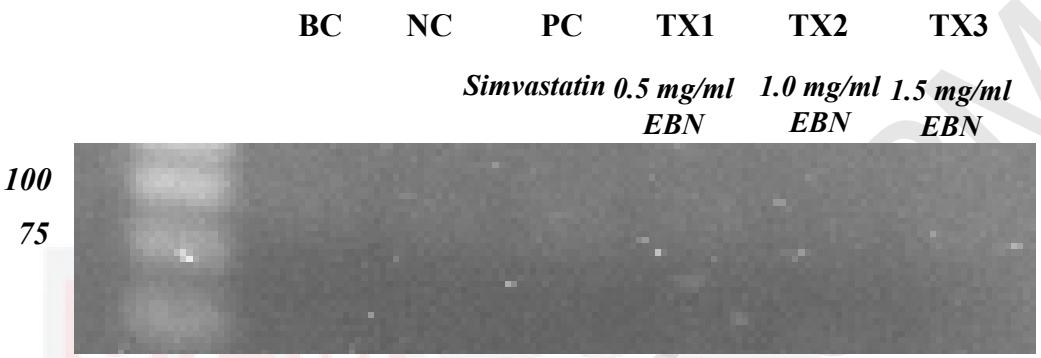

Figure 5: Analysis of PCSK9 gene expression in HepG2 cells using RT-PCR. Monolayer culture of HepG2 cells were grown as described under "Cell Culture". Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The PCR products predicted to be 87 bp, were resolved on SYBR Safe (Invitrogen) stained 2.0% agarose gel alongside a 25bp DNA ladder (Promega).

PCSK9 secretes protease which will lead to binding to and degradation of the LDLR. Its expression is controlled by SREBPs and simvastatin treatment will increase PCSK9 expression in HepG2 cells. Based on the result shown above, PCSK9 gene expression in Treatment 1, 2 and 3 were downregulated as compared to the controls. It is suggested that EBN extract supplementation will help to reduce PCSK9 gene expression in HepG2 mammalian cells which is a good indicator as high level of PCSK9 will lead to LDLR degradation, hence, leading to increased amount of circulating LDL in the blood.

4.6 3-Hydroxy-3-Methylglutaryl-Coa Reductase (HMGCR)

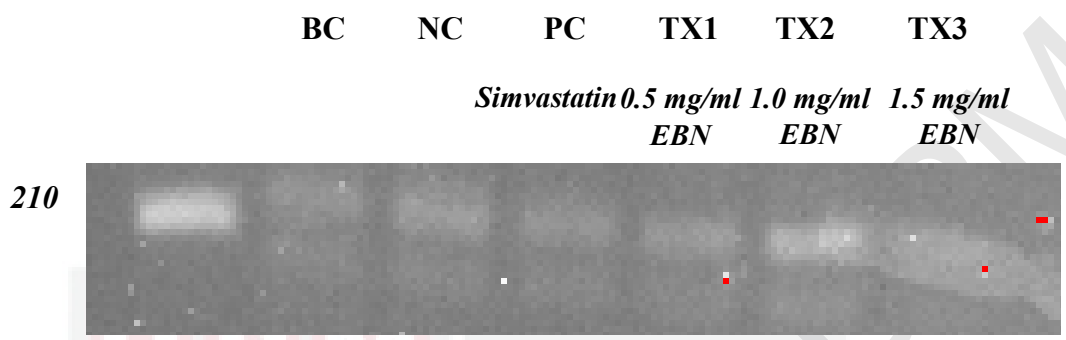

Figure 6: Analysis of HMGCR gene expression in HepG2 cells using RT-PCR. Monolayer culture of HepG2 cells were grown as described under "Cell Culture". Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, German) according to the manufacturer's protocol. The PCR products predicted to be 210 bp, were resolved on SYBR Safe (Invitrogen) stained 1.5% agarose gel alongside a 1kb DNA ladder (Promega).

HMGCR is the rate limiting enzyme for cholesterol biosynthesis which is primarily regulated via negative feedback mechanism mediated by sterols and non-sterol metabolites. Based on the result shown above, HMGCR gene expression level have been slightly upregulated with supplementation of EBN extract and exogenous lipid in comparison to the controls especially in Treatment 2 with EBN concentration of 1.0 mg/ml.

CHAPTER 5

DISCUSSION

Statins drugs target hepatocytes and inhibit HMGCR which converts HMG-CoA into mevalonic acid, a type of cholesterol precursor. Simvastatin, Lovastatin, Fluvastatin and Atorvastatin are a few examples of most commonly used anti-cholesterol. Statins can be classified according to how they are obtained, the liver metabolism, physio-chemical properties and its specific activity. In this study, Simvastatin is a drug of choice for the Positive Control.

ACAT2 protein expression can be found in only 2 types of cells, namely enterocytes and the endoplasmic reticulum of the hepatocytes based from immunofluorescence analysis (Lee et al., 2000; Parini et al., 2004). The main function of ACAT2 in hepatocyte is to provide esterified cholesterol for the incorporation into VLDL as well as cholesteryl ester for cytoplasmic lipid droplets whereas ACAT2 in enterocyte, has been hypothesized to play an important role in cholesterol absorption and esterification of cholesterol into the circulation.

Cholesterol is hydrophobic thus incorporation into the chylomicron, a more hydrophilic form, will solve this problem thus cholesteryl ester can be easily transported around the body via the blood stream. The role of ACAT2 is further demonstrated in studies using ACAT2 knockout mice whereby there are evidence of resistance to hypercholesterolemia, reduced cholesterol gallstones, and a lowered exogenous cholesterol absorption from the intestines (Lawrence et al., 2005).

Based from the result, the cells treated with EBN extract in comparison to the controls have been shown to have significant upregulation of ACAT2 gene expression. As for comparison with the Positive Control containing Simvastatin drug, EBN extract supplementation have been shown to produce a more desirable result.

The second gene tested is DGAT2 which is the major TAGs synthesizing enzyme located in ER. This gene is important for catalyses of TAGs for incorporation in lipid droplet expansion with diacylglycerol and fatty acyl-CoA as substrates. Stone et al. (2004) in their study "Lipopenia and skin barrier abnormalities in DGAT2-deficient mice", reported DGAT2-deficient mice will die shortly after birth due to no TAG present (Stone et al., 2004) whereas overexpression or upregulation of DGAT2 in cell culture resulted in massive increased in intracellular lipid droplets (Monetti et al., 2007; Stone et. al., 2004). However, based from this study, there are no significant differences in term of the level of DGAT2 gene expression with EBN extract supplementation and exogenous lipid concentrate in HepG2 mammalian cells in comparison with the controls.

Next, the gene of interest is LDLR, the famous cell-surface glycoprotein which plays a very important role in maintaining the blood cholesterol level. This receptor will react with Apolipoprotein B specifically located on LDL and facilitates the internalization and degradation of LDL into the cell hence reducing the amount of circulating cholesterol in the blood. LDL is generally known as the 'bad cholesterol' as it causes Atherosclerotic Cardiovascular Disease (ASCVD).

Familial Hypercholesterolaemia (FH) is an autosomal co-dominant mutation in LDLR gene where LDL receptors function are decreased LDL leading to a markedly increased concentration of circulating LDL particles and the cholesterol carried by those particles (Brian et al., 2017). EBN has a good potential in reducing blood cholesterol as supplementation with EBN extract increases LDLR expression and the effect is much more significant compared to Simvastatin drug.

The predominant site for PCSK9 production is hepatocytes along with other sites such as intestines and kidney (Rashid et. al., 2005; Zaid et. al, 2008). PCSK9 interacts directly with LDLR and its major function is regulation of cholesterol homeostasis via degradation of LDLR (Seidah, 2009; Seidah, 2012). Apart from lipid metabolism, PCSK9 has been shown to play a role in glucose homeostasis (Mbikay et al., 2010), and liver regeneration and susceptibility to hepatitis C virus infection. (Farnier, 2011; Seidah et al., 2013).

Overexpression of PCSK9 induced and excess of atherosclerosis (Denis et al., 2012). The gene expression is mainly modulated by intracellular cholesterol concentrations and activation of SREBP2. Inhibition of PCSK9 can be achieved with the use of human monoclonal antibodies (mAbs) such as Alirocumab and Evolocumab which will bind to LDLR and prevent its degradation. PCSK9 inhibitors will reduce atherogenic lipoproteins and at the same time enhancing Statin efficacy (Farnier, 2014). Based from this study, it is proposed that EBN extract supplementation will help to reduce PCSK9 gene expression in HepG2 mammalian cells which has the same effect as PCSK9 inhibitors.

CHAPTER 6

6.1 CONCLUSION

Among the six genes related to lipid signalling pathways tested, EBN extract have been found to have significant effects on ACAT2 and LDLR genes. ACAT2 gene is responsible for the synthesis of sterol ester where it will be incorporated in lipid droplets for lipid storage whereas LDLR gene is the most important key player in internalization of LDL from the blood stream into the cells. Hence, upregulation of LDLR gene will increase the number of LDL receptors on the outer surface of the cells thus reducing the amount of circulating LDL (bad cholesterol) in the blood which is very beneficial so as to lower the risk of atherosclerosis or cholesterol deposition in the vessels. As for PCSK9 gene, although the result is considered questionable due to its clarity, EBN extract supplementation might have a positive effect in reducing PCSK9 expression. Further research can be done to look into deeper the significant of EBN extract towards genes expression.

6.2 RECOMMENDATIONS

I would like to propose a recommendation to extend/provide a longer period for final Year Project. Research-based projects as such in this project require a longer period of time in order to produce a more reliable and satisfactory result and at the same time minimizing technical error. As for recommendation related to my project, a higher cell confluency, approximately 80-100%, is needed in order to achieve a higher RNA concentration for PCR study.

REFERENCES

Aswir AR, Wan Nazaimoon WM. Effect of edible bird's nest on cell proliferation and tumor necrosis factor- alpha (TNF-α) release in vitro . Int Food Res J 2011;18:1123-1127.

Bartz, R., Li, W. H., Venables, B., Zehmer, J. K., Roth, M. R., Welti, R., Anderson, R. G., Liu, P. and Chapman, K. D. (2007). Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic. J. Lipid Res. 48, 837-847.

Brian A. Ference, Henry N. Ginsberg, Ian Graham, Kausik K. Ray, Chris J. Packard, Eric Bruckert, Robert A. Hegele, Ronald M. Krauss, Frederick J. Raal, Heribert Schunkert, Gerald F. Watts, Jan Borén, Sergio Fazio, Jay D. Horton, Luis Masana, Stephen J. Nicholls, Børge G. Nordestgaard, Bart van de Sluis, Marja-Riitta Taskinen, Lale Tokgözoğlu, Ulf Landmesser, Ulrich Laufs, Olov Wiklund, Jane K. Stock, M. John Chapman, Alberico L. Catapano; Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel, European Heart Journal, Volume 38, Issue 32, 21 August 2017, Pages 2459–2472, https://doi.org/10.1093/eurheartj/ehx144

Guo C, Takahashi T, Bukawa W, Takahashi N, Yagi H, Kato K, et al. Edible bird's nest extract inhibits influenza virus infection. Antiviral Res 2006;70:140-146

Ibrahim SH, Teo WC, Baharun A. A study on suitable habitat for swiftlet farming. UNIMAS E-J Civil Eng. 2009; 1(1): 1–7.

Kang N, Hails CJ, Sigurdsson JB. Nest construction and egg-laying in edible nest swiftlets Aerodramus spp. and the implications for harvesting. Int J Avian Sci. 1991; 133(2): 170–177.

Kong YC, Keung WM, Tip TT, Ko KK, Tsao SW, Ng MH. Evidence that epidermal growth factor is present in Swiflet's (Collocalia) nest. Comparat Biochem Physiol 1987;87:221-226.

Lagor, William & S Millar, John. (2010). Overview of the LDL receptor: Relevance to cholesterol metabolism and future approaches for the treatment of coronary heart disease. Journal of Receptor, Ligand and Channel Research. 3. 1-14. 10.2147/JRLCR.S6033.

Lawrence L. Rudel, Richard G. Lee and Paolo Parini. 2005. ACAT2 is A Target for Treatment of Coronary Heart Disease Associated with Hypercholesterolaemia. Arterioscler Thromb Vasc Bio. 2005; 25:1112-1118

Lee R G, Willingham M C, Davis M A, Skinner K A, Rudel L L. Differential expression of ACAT1 and ACAT2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. J Lipid Res. 2000;41: 1991–2001.

Lim C. Make millions from swiftlet farming: a definitive guide. Selangor: True Wealth. 2006.

Lim CK & Oswald BK (2004). Swiflets management in Sarawak; conserving wild colonies Malaysia: In: Proceedings Edible-Nest Swiflets Management in Asia, pp: 35-46.

Looi, Q. H., Amin H., Aini, I., Zuki, M., Omar, A. R. (2017). De novo transcriptome analysis shows differential expression of genes in salivary glands of edible bird's nest producing swiftlet. *BMC Genomics.* https://doi.org/10.1186/s12864-017-3861-9

Matsukawa N, Matsumoto M, Bukawa W, Chiji H, Nakayama K, Hara H, et al. Improvement of bone strength and dermal thickness due to dietary edible bird's nest extract in ovariectomized rats. Biosci Biotechnol Biochem 2011;75:590-592.

Marcone MF. Characterization of the edible bird's nests the "Caviar of the East". Food Res Int. 2005; 38(10): 1125– 1134.

Mbikay M, Siois F, Mayne J, et al. 2010. PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities. FEBS Lett; 584:701-6

N. Krahmer, R.V. Farese Jr., T.C. Walther, Balancing the fat: lipid droplets and human disease, EMBO Mol. Med. 5 (2013) 973–983.

Ng MH, Chan KH, Kong YC. Potentiation of mitogenicity response by extracts of swiftlet's (Collocalia) nest. Biochem Int 1986;13:521-531

Parini P, Davis M, Lada AT, Erickson SK, Wright TL, Gustafsson U, Sahlin S, Einarsson C, Eriksson M, Angelin B, Tomoda H, Omura S, Willingham MC,

Rudel LL. ACAT2 is localized to hepatocytes and is the major cholesterol esterifying enzyme in human liver. Circulation. 2004; 110:2017–2023

Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA, Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc Natl Acad Sci USA 2005; 102: 5374-5379 [PMID: 15805190 DOI: 10.1073/ pnas.0501652102]

Seidah NG, Sadr MS, Chretien M, et al. The multifaceted proprotein convertases: their unique, redundant, complementary, and opposite functions. J Biol Chem;288:21473-81

Seidah NG. 2009. PCSK9 as a Therapeutic Target of Dyslipidaemia. Expert Opin Ther Targets; 13:19-28

Seidah NG, Prat A. 2012. The Biology and Therapeutic Targeting of the Proprotein Convertase Neural Apoptosis-Regulated Convertase. Nat Rev Drug Discov;11:367-83

Shah, Syed Waliullah & Aziz, Azlin. (2014). Morphology of the lingual apparatus of the Swiftlet, Aerodramus fuciphagus (Aves, Apodiformes, Apodidae). Journal of Microscopy and Ultrastructure. 2. 100-103. 10.1016/j.jmau.2014.02.006.

Stone, S. J., Myers, H. M., Watkins, S. M., Brown, B. E., Feingold, K. R., Elias, P.M., and Farese, R.V.,Jr. (2004) Lipopenia and skin barrier abnormalities in DGAT2-deficient mice.J.Biol.Chem.279,11767–11776

Wong, R. S. Y. (2013) Edible Bird's Nest: Food or Medicine? *Chinese Journal of Integrative Medicine*, 19, 643-649. doi: 10.1007/s11655-013-1563-y

Youzhi Jin, Pamela J. McFie, Shanna L. Banman, Curtis Brandt, and Scot J. Stone 2014. Diacylglycerol Acyltransferase-2 (DGAT2) and Monoacylglycerol Acyltransferase-2 (MGAT2) Interact to Promote Triacylglycerol Synthesis. The Journal of Biological Chemistry Vol. 289, NO. 41, pp. 28237–28248.

Zainal Abidin F, Hui CK, Luan NS, Mohd Ramli ES, Hun LT, Abd Ghafar N. Effects of edible bird's nest (EBN) on cultured rabbit corneal keratocytes. BMC Complement Altern Med 2011;11:94.

Zaid A, Roubtsova A, Essalmani R, Marcinkiewicz J, Chamberland A, Hamelin J, Tremblay M, Jacques H, Jin W, Davignon J, Seidah NG, Prat A. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. Hepatology 2008; 48: 646-654 [PMID: 18666258 DOI: 10.1002/hep.22354]

Zhang M, Wang D, Wang J. The effects of the Zhenzhu Yanwo extracts on animal function. Pharm Biotech 1994;1:49-51.

APPENDICES

APPENDIX I

LIST OF MATERIALS

1. Advance Minimum Essential Media (AMEM) (Gibco™ Cat. No.:12492013)
2. Fetal Bovine Serum (FBS) (Gibco™ Cat. No.:10438018)
3. Pen-Strep (Gibco™ Cat. No.: 15140122)
4. Fungizone (Gibco™ Cat. No.:15290018)
5. Trypsin-EDTA (Gibco™ Cat. No.: 25300062)
6. Trypan blue (Gibco™ Cat. No.: 15250061)
7. 70% ethanol in water (SIGMA-ALDRICH Product no.: R8382)
8. 1M Phosphate buffered saline (PBS)
9. Low Density Lipoprotein (Invitrogen™ Cat. No.: L3486)
10. Cholesterol Concentrate (Gibco™ Cat. No.: 12531018)
11. Simvastatin (Calbiochem-Merck Co)
12. ACAT2_R Primer (IDTDNA® Ref. No.: 98857030)
13. ACAT2_F Primer (IDTDNA® Ref. No.: 98857029)
14. HMGCR_F Primer (IDTDNA® Ref. No.: 99340633)
15. HMGCR_R Primer (IDTDNA® Ref. No.: 99340635)
16. DGAT2_F Primer (IDTDNA® Ref. No.: 98527515)
17. DGAT2_R Primer (IDTDNA® Ref. No.: 98527520)
18. LDL_F Primer (IDTDNA® Ref. No.: 98527512)
19. LDL_R Primer (IDTDNA® Ref. No.: 98527517)

20. SREBP2_F Primer (IDTDNA® Ref. No.: 100170839)
21. SREBP2_R Primer (IDTDNA® Ref. No.: 100170841)
22. PCSK9_F Primer (IDTDNA® Ref. No.: 1001170840)
23. PCSK9_R Primer (IDTDNA® Ref. No.: 100170842)
24. IKA® VORTEX GENIUS 3
25. SYBR™ Safe DNA Gel Stain (Thermo Scientific™ Cat No.: S33102)
26. Access RT-PCR Systems (Promega® Cat No.: A1280)
27. 25 bp DNA Step Ladder (Promega Cat. No.: G4511)
28. 1kb DNA Ladder (Promega Cat. No.: G5711)
29. BCA Protein Assay kit (Thermo Scientific™ Cat No.: 23225)
30. BIO-RAD Molecular Imager ® Gel Doc™ XR+ with Image Lab ™ Software
31. PowerPac™ Basic
32. Kimberly-Clark Corporation 34155 Kimwipes tissue
33. PARAFILM® M sealing film
34. Eppendorf™ 5702R centrifuge
35. ESCO CelCulture CO2 incubator
36. 96-well microplate (Greiner Bio-one Cat no.: 650 001)
37. TECAN infinite M200 pro spectrophotometer
38. MDF-U76V-PE VIP ULT Upright Freezer
39. Freezing container, Nalgene® Mr. Frosty (SIGMA-ALDRICH Product no.: C1562)
40. Mr. Frosty™ Freezing Container (Thermo Scientific™ Cat. No.: 5100-0001)
41. Memmert Waterbath WNB 45
42. Haemocytometer (MARIENFELD Cat. No.: 0680010)

43. Inverted microscope (OLYMPUS CKX41)
44. Eppendorf tubes (Eppendorf™ Cat. No.: 022431021)
45. PIPETMAN® P P1000 micropipette (F1123602) and tips (F171703)
46. PIPETMAN® P P200 micropipette (F123601)) and tips (F171503)
47. PIPETMAN® P P20 micropipette (F 123600)) and tips (F171503)
48. PIPETMAN® P P2 micropipette (F144801) and tips (F171103)
49. Cryogenic vials (Corning® Product #430488)
50. 6-well plate (Corning® Product #3516)
51. T75 flask (Corning® Product #430641)
52. T25 flask (Corning® Product #430639)
53. 50 ml round bottom glass tube (Corning® Product #842250)
54. 250ml storage bottles (Corning® Product #430281)
55. 1ml serological pipette (Corning® Product #4485)
56. 2ml serological pipette (Corning® Product #4486)
57. 5ml serological pipette (Corning® Product #4487)
58. 10ml serological pipette (Corning® Product #4488)
59. 15ml centrifuge tube (Corning® Product #430791)
60. 50ml centrifuge tube (Corning® Product #430829)
61. 3 ml syringe (Terumo® Product #SS-03L)
62. Memmert UF110 lab oven
63. Raw EBN
64. Forceps
65. Glass slide
66. Mortar

67. Pestle

68. Cold acetone

69. Distilled water

APPENDIX II

LIST OF PRIMERS USED

No	GENES	FORWARD PRIMER	REVERSE PRIMER	ASCENSION NUMBER
1.	ACAT2	GCCTCAGACAAT ACAATGG	AAACACGTAACGA CAAGTCC	NM_003101.5
2.	DGAT2	AGTGGCAATGCT ATCATCAT	GAGGCCTCGACCA TGGAAGAT	NM_001253891.1
3.	LDLR	CCCCGCAGATCA ACCCCCACTC	AGACCCCCAGGCA AAGGAAGACGA	NM_000527.4
4.	HMGCR	GGGACCAACCTA CTACCTC	GTCGAAGATCAAT TTACAA	
5.	SREBP2	AACGGTCATTCA CCAGGTC	GGCTGAAGAATAG GAGTTGCC	XR_001755278.1
6.	PCSK9	ATCCACGCTTCC TGCTGC	CACGGTCACCTGC TCCTG	NM_174936.3