THE EFFECT OF BENJAKUL WATER EXTRACT ON PANCREAS IN RATS FED A HIGH-FAT DIET

BY

MISS KEVALIN VONGTHOUNG

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
THE DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES
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ENTITLED

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ABSTRACT

Benjakul is a name of the medicinal formula widely used in Thai traditional medicine. The formula is composed of five species of medicinal plants, *Piper sarmentosum* Roxb. (wild betal leaf bush), *Piper retrofractum* Vahl. (long pepper), *Piper interruptum* Opiz. (Pepper wood), *Plumbago indica* Linn. (rose-color lead wood) and *Zingiber mekongense* Gagnep. (ginger). Decoction of Benjakul has been used as a kind of adaptogenic agent. The certain formula of Benjakul has been prescribed to reduce hyperglycemia. Since one of the symptoms found in metabolic syndrome is the prediabetes state defined as mild hyperglycemia and glucose intolerance, it is interesting to study the effect of Benjakul on this situation in metabolic syndrome. The study extends its intention, furthermore, to evaluate its effect on the other relating biochemical disturbance and pathological signs of tissue, especially pancreas. The study was done in Sprague Dawley rats fed with a high fat diet. The investigation includes the determination of blood parameters associated with the syndrome, organ weight and the effect on the specific organ, pancreas. The later includes the effect of Benjakul on insulin secreting function in relating to blood parameters and some pathological effects of the pancreas. Decoction of Benjakul was freeze-dried. High-fat fed rats were gavaged with 41.3 mg/kg rat weight (HFB1) or 413 mg/kg rat weight (HFB10). The study was compared to rats fed with high-fat

alone (HF) and the control, fed with standard chow. The effect of the whole plant of *Piper sarmentosum* Roxb., a single herb remedy, was studied in comparison to the formula as Benjakul. Metformin was used as a positive control since it was usually prescribed in metabolic syndrome. The results showed abdominal and epididymal fat weights, adipocyte size, and total cholesterol and low-density lipoprotein-cholesterol (LDL-C) levels were decreased, whereas serum high-density lipoprotein-cholesterol (HDL-C) levels were increased in the short-term HFB1 group in comparison with the HF group. Short-term HFB10 group showed a significant decrease in abdominal fat weight, adipocyte size and serum triglyceride levels when compared with the HF group. The same trends were observed in HFW and HFM groups. Although there were no significant differences in total cholesterol, LDL-C and HDL-C levels between the control and HFB10 groups. Consistently, intermediate-term treatment with low dose of BWE reduced abdominal fat weight and adipocyte size. For the effects of BWE on glucose homeostasis parameter, all short-term treatments had no effect on the FBG and fasting serum insulin levels, in high-fat diet-fed rats. Unexpectedly, the intermediate-term HFB1 group had significantly increased AUC, whereas the HF group did not have any significant change compared with the control group. When focus on the effects of BWE on pancreatic abnormalities, in the short-term experiment, low and high dose BWE increased the expression of pancreatic insulin signaling gene, insulin receptor substrate-2 (IRS-2), but had no effect on glucose-sensing genes, glucose transporter-2 (GLUT-2) and glucokinase (GK). Short-term treatments with WWE and metformin significantly prevented the decrease of IRS-2 and GLUT-2 mRNA levels in high-fat diet-fed rats. In contrast to the short-term study, intermediate-term treatment with BWE suppressed the expression of pancreatic IRS-2, GLUT-2 and GK mRNA. Notably, histological study of pancreas from all treatment groups showed a reduction of vacuoles formation in pancreatic acinar cells but had no effect on pancreatic senescence. In addition, only the intermediate-term HFB1 group showed a significant decrease in the pancreatic nuclear factor-kappa B p65 (NF-κB p65) mRNA levels. In conclusion, the results suggested that BWE has a protective effect against pancreatic abnormalities in rats fed-high fat diet. The mechanisms may be related to up-regulation of insulin signaling gene and inhibition of vacuole accumulation in the pancreas. It is compatible with the

compensation mechanism of pancreas in early stage of diabetes, same as the compensation mechanism in metabolic syndrome. Since intermediate-term treatment of BWE may not appropriate. Therefore, BWE may appropriate for short-term used, same as metformin in metabolic syndrome treatment. Further pharmacological evaluations are required to identify and isolate the active compounds in BWE for elucidating their mechanisms of active on lipid metabolism.

Keywords: Obesity, Metabolic syndrome, Glucose tolerance, Insulin resistance, Cellular senescence

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
°C	Degrees Celsius
α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
μ	Mu
μg	Microgram
μL	Microliter
μm^2	Square micrometers
μm	Micrometer
∞	Infinity
4-AAP	4-aminoantipyrine
ALT	Alanine aminotransferase
a.m	Ante meridian
Akt	Protein kinase B, also known as Akt
AMPK	AMP-activated protein kinase
ANOVA	One-way analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC-G	Area under the curve of blood glucose levels
BHT	Butylate hydroxytoluene
BJK	Benjakul
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight

BWE	Benjakul water extract
cAMP	Cyclic adenosine monophosphate
CEPT	Cholesteryl ester transfer protein
cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
CT	Carnitine: acylcarnitine translocase
DAB	Diaminobenzidine tetrachloride
DAG	Diacylglycerol
DAP	Dihydroxyacetone phosphate
DBP	Diastolic blood pressure
DEPC-treated water	Diethylpyrocarbonate-treated water
DGAT	Diacylglycerol acyltransferase
dL	Deciliter
dNTPs	Deoxyribonucleotide triphosphates
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Distilled water
dUTPs	Deoxyuridine triphosphate
EC50	Half maximal effective concentration
EGIR	European group for the study of Insulin Resistance
ELISA	Enzyme-linked immunosorbent assay
ESPA	*N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine
FA	Fatty acid
FABP	Fatty acid binding protein
FACS	Fatty acyl CoA synthase
$FADH_2$	Flavin adenine dinucleotide
FBG	Fasting blood glucose
FFA	Free fatty acid
FoxO	Forkhead box protein O
G-1-P	Glycerol-1-phosphate
G-3-P	Glycerol-3-phosphate

G-6-P	Glycerol-6-phosphate
GAE	Gallic acid equivalents
GK	Glucokinase
GLUT-2	Glucose transporter-2
GLP1	Glucagon-like peptide 1
GLP1R	Glucagon-like peptide 1 receptor
GPAT	Glycerol-3-phosphate acyltransferase
GPR40	G-protein-coupled receptor 40
GSIS	Glucose-stimulated insulin secretion
h	hour
H & E	Hematoxylin and eosin
HDL-C	High-density lipoprotein-cholesterol
HF	High-fat diet
HFB1	High-fat diet co-fed with low-dose of Benjakul water extract
HFB10	High-fat diet co-fed with high-dose of Benjakul water extract
HFM	High-fat diet co-fed with metformin
HFW	High-fat diet co-fed with wild betal leaf bush water extract
IκB	Inhibitor of κB
ICAM-1	Intracellular adhesion molecule-1
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IgG	Immunoglobulin G
IGT	Impaired glucose tolerance
IHC	Immunohistochemistry
IKK	Inhibitor of nuclear factor κB kinase
IR	Insulin resistance
IRS-2	Insulin receptor substrate-2
JNK	c-Jun N-terminal kinase

kcal	Kilocalories
kg	Kilogram
L	Liter
LDH	Lactate dehydrogenase
LDL-C	Low-density lipoprotein-cholesterol
LPL	Lipoprotein lipase
MCAD	Medium-chain acyl CoA dehydrogenase
MCD	Malonyl CoA decarboxylase
MCP-1	Monocyte chemoattractant protein-1
M-CSF-1	Macrophage-colony stimulating factor-1
mg	Milligram
MGB	Minor groove binder
MIF	Macrophage migration inhibitory factor
min	Minutes
mL	Milliliter
mm^2	Square millimeters
mmol	Millimole
mRNA	Messenger ribonucleic acid
mU	Milliunit
MW	Molecular weight
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
NCEP ATPIII	National Cholesterol Education Programme Adult Treatment Panel III
NEFA	Non-esterified fatty acid
NF-κB p65	Nuclear factor NF-κB p65 subunit
NFQ	Nonfluorescent quencher
ng	Nanogram
NIK	Nuclear factor NF-κB inducing kinase
nm	Nanometer

NR1C	The group C in the subfamily 1 of the superfamily of nuclear hormone receptors
OGTT	Oral glucose tolerance test
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 3,4 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RQ	Relative quantitation
RT	Reverse transcription
sec	Second
SA-β-gal	Senescence-associated β-galactosidase
SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M	Standard error of the means
SH2	Src homology 2
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.1% Tween-20
TCA	Tricarboxylic acid
TG	Triglyceride
TNF-α	Tumor necrosis factor-α

Total-C	Total cholesterol
U	Unit
UDG	Uracil-DNA glycosylase
VLDL	Very low-density lipoprotein-cholesterol
WHO	World Health Organization
WWE	Wild betal leaf water extract

CHAPTER 1
INTRODUCTION

1.1 Research problem

Obesity has been appeared as one of the most significant public health problem in Western industrialized nations and developing countries including Thailand (Aree Kantachuvessiri, 2005; Haslam & James, 2005; Hedley et al., 2004; Hofbauer, Nicholson, & Boss, 2007; Prentice, 2006; Yach, Stuckler, & Brownell, 2006; Yoon et al., 2006). More than 1.9 billion adults aged equal to or greater than 18 years people were overweight. The worldwide prevalence of obesity was greater than doubled between 1980 to 2014 (World Health Organization, 2015). Obesity, particularly abdominal or visceral or central obesity is a relevant predictor of chronic non-communicable diseases derived from abnormal energy metabolism such as gained threat for type 2 diabetes mellitus (T2DM) (Bray, Clearfield, Finte, & Nelinson, 2009). Visceral adipose tissue, in particular, can secrete adipokines that can induce insulin resistance and inflammation (Fulop, Tessier, & Carpentier, 2006). Abdominal obesity is the major determinant and the most prevalent manifestation of the metabolic syndrome (Monteiro & Azevedo, 2010). Insulin resistance was defined as the inability of purpose organs such as, liver, muscle, and fat tissues to reply effectively to insulin stimulation (Pittas, Joseph, & Greenberg, 2004). Therefore, during the development of insulin resistance, the body compensates by increasing insulin secretion (Pittas, Joseph, & Greenberg, 2004). In order to maintain normal blood glucose, due to increased body mass, the body compensates by hypersecretion of insulin and increased insulin synthesis, resulting in increased β-cell mass (Plentki & Nolan, 2006).

Cellular senescence is the state of unalterable cell-cycle inhibit (Salama, Sadaie, Hoare, & Narita, 2014). It is involved in the development of many pathological features including the decreases in pancreatic mass and insulin synthesis. Animal studies of metabolic syndrome have been suggested that pancreatic senescence could occur as a consequence of an increase in islet cell proliferation (Sone & Kagawa, 2005). Increased action of nuclear factor-κB (NF-κB), was found in

the pancreatic tissues of rats fed with a high-cholesterol diet (Czakó, Szabolcs, Vajda, Csáti, Venglovecz, & Rakonczay, 2007). Furthermore, a previous study has been proposed that the mobilization of NF-κB signaling pathway was associated with the induction of senescent process (Tilstra, Robinson, Wang, Gregg, Clauson, & Reay, 2012).

Several studies have been suggested that defective insulin signaling in the pancreas is an influential event in the development of metabolic syndrome and diabetes that could be linked to the down-adjustment of genes associated in insulin signaling, such as insulin receptor substrate-2 (IRS-2) gene (Xiao, Gregersen, Pedersen, & Hermansen, 2002). IRS-2 is an important branch of insulin signaling path that has been suggested to amusement a character character in the adjustment of pancreatic mass and insulin synthesis. Moreover, the upregulation of IRS-2 in β-cells could prevent the progression of diabetes in mice (Hennige, Burks, Ozcan, Kulkarni, Ye, & Park, 2003). Impaired glucose sensing in β- and α-cells is also recognized as an adverse process that inhibits glucose-stimulated insulin secretion (GSIS), outstanding to impaired glucose tolerance (Ahrèn, 2009). It is well established that both GLUT-2 and glucokinase (GK) function as a glucose sensor for stimulating insulin secretion. Previous studies have been shown the down-regulations of both these genes in the pancreatic tissues of animals fed with a high-fat diet (Reimer & Ahren, 2002; Gremlich, Bonny, Waeber & Thorens, 1997).

Benjakul is a name of the Thai traditional medicine. It composed of five species of medicinal plants, namely, *Piper retrofractum* Vahl. (long pepper) fruit, *Piper sarmentosum* Roxb. (wild betel leaf bush) root, *Piper interruptum* Opiz. (pepper wood) stem, *Plumbago indica* Linn. (rose-color lead wood) root, and *Zingiber mekongens*e Gagnep. (ginger) rhizome (Ministry of Education, 1999). It has been used for practicing balanced health in Thai traditional medicines and as an adaptogen in the lists of the National Drug List of Herbal Medicinal Products A. D. 2006 (National List of Essential Medicines, 2013) (APPENDIX A). It has been widely used to be adaptogen for cancer treatment by folk doctors in the Southern region of Thailand (Itharath, Singchangchai, & Rattanasuwan, 1999). It has been shown to have no poisonous, either intensely or constantly, in both tested creatures and mans (Chaovalitthamrong, Attawit, Rugsamun & Junpen, 1996; Amorndoljai, Kietinun, &

Somparn, 2011). The pharmacokinetics of piperine from *Piper retrofractum* Vahl., the major active component of Benjakul ethanol extract formulation, was dose-dependent (Jumpa-ngern, Kietinun, Sakpakdeejaroen, Cheomung, & Na-Bangchang, 2013). However, the results of Benjakul water extract (BWE) on pancreas have not yet been investigated in high-fat diet fed rats. Accordingly, the principal purpose of the existing examine was to verify the result of BWE on the modulation of glucose tolerance and insulin resistance in high-fat diet-induced obese rats.

1.2 Hypothesis

Based on Thai folk medicine and previous studies, the present study therefore hypothesized that the BWE treatment could attenuate metabolic risk parts and pancreatic abnormalities in a metabolic syndrome rat model.

1.3 Objectives

The objects of the study are to determine the effects of the Benjakul extraction on the prevention of pancreatic abnormalities in a rat model of metabolic syndrome derived from receiving a high-fat diet. The general objective of this study is to investigate the effect of Benjakul on the precise molecular modulation of rat pancreatic abnormalities via the improvement of abdominal fat accumulation, dyslipidemia and insulin resistance in short and intermediate high-fat diet. Therefore, our specific objectives of the study are as following:

1.3.1 To study the effect of Benjakul on abdominal fat and epididymal fat weight and adipocyte size.

1.3.2 To examine the effect of Benjakul on serum lipid profile.

1.3.3 To investigate the effect of Benjakul on glucose homeostatic parameters.

1.3.4 To investigate the effect of Benjakul on liver and kidney function test.

1.3.5 To investigate the effect of Benjakul on pancreatic mass, histopathology and senescence.

1.3.6 To investigate the effect of Benjakul on pancreatic IRS-2, GLUT-2, GK and NF-κB p65 gene expressions.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Obesity

2.1.1 Definition of obesity

Obesity can be described as an abnormal overabundance of body fat accumulation and detrimental contributor to human health. Although knowledge of the role of genetic factors in obesity is elevating, the fast increase in prevalence of overweight and obesity throughout the world indicates that environmental factors such as high-fat diet, high-carbohydrate diet and tangible inactivity are the major contributor of this epidemic. Pathogenesis of obesity can be explained as a maintained disequilibrium between energy intake and energy consumption in the body, may result from either on nutrient overload relative to dissipative capacity, a decreased in energy efflux ability to limit increased energy influx, or combination of both situations. Disruption of this balance potentially can affect increase fat deposits in adipose tissue, especially abdominal fat accumulation and non-adipose tissue (ectopic fat accumulation).

The adipose tissue is not only concerned in major source of energy saving but is also an endocrine organ that produces and secretes a large enumerate of bioactive substances, known as adipokines, that have regulatory activities of metabolic homeostasis, feeding behavior, blood pressure, coagulation and inflammatory responses in our body such as free fatty acid (FFA), leptin, resistin, retinol binding protein 4 (RBP4) and adiponectin. The dysregulated generation and releasing of these substances, caused by excess adiposity, abnormal adipocyte structure and adipocyte dysfunction, may have a character in the pathogenesis of diverse diseases through changed metabolic and immunoinflammatory responses (metainflammation) (Galic, Oakhill, & Steinberg, 2010; Harwood, 2012).

Under situations of chronic overnutrition that results from a combining of gained caloric intake and declined energy expenditure conduces to expanding adipose tissue mass (adipocyte hypertrophy and hyperplasia), beginning a conditions of cellular stress and stimulation of pro-inflammatory pathways, especially nuclear

factor-kappa B (NF-κB) signaling, C-Jun N-terminal kinase (JNK) and endoplasmic reticulum stress (ER stress). This results in upregulated adipocyte production of pro-inflammatory adipokines for example monocyte chemoattractant protein-1 (MCP-1) that recruits monocytes-macrophages and other immune cells infiltration into the adipose tissue and aggravates the inflammatory response. These macrophages are activated to release inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) which interfere the antilipolytic effect of insulin. In adipocytes, insulin resistance leads to an elevated efflux of FFA from adipocytes to circulation and other tissues such as liver, muscle, pancreatic p-cell, hypothalamus, as well as to the heart and blood vessel. This active adipocytes- macrophages crosstalk leading to dysregulated secretion of a variety of adipokines and massive amounts of intracellular lipid and its toxic metabolites accumulation (lipotoxicity) such as triglyceride, diacylglycerols (DAG) and ceramides result in metabolic derangement, insulin resistance, inflammation, oxidative stress and multiorgan dysfunction on numerous ectopic tissues. These systemic dysfunction that extends the danger for generation of the metabolic syndrome (dyslipidemia and abnormal glucose homeostasis) and type 2 diabetes (Attie & Scherer, 2008; Cusi, 2010; Gregor & Hotamisligil, 2011; Harwood, 2012; Karastergiou & Mohamed-Ali, 2010; Lionetti et al., 2009; Lottenberg, da Silva Afonso, Lavrador, Machado, & Nakandakare, in press; Maury & Brichard, 2010; Muoio & Newgard, 2006; Samuel, Petersen, & Shulman, 2010; Snel et al., 2012; Unger, Clark, Scherer, & Orci, 2010). The pathogenesis of obesity and its related disease are shown in figure 2.1.

2.1.2 Visceral obesity

Waist circumference is included in the meaning of the metabolic syndrome (Alberti & Zimmet, 1998).The ratio of waist-to-hip circumferences (WHR) measures the degree of central (visceral or abdominal) vs. peripheral (subcutaneous) adiposity. Visceral fat is a major risk for metabolic disease, whereas peripheral fat appears to be benign to metabolic complications (Timar, 1999).

Accompanied by increases in intra-abdominal or visceral adipose tissue, a higher degree of current of adipose tissue-derived FFA to the liver via the splanchnic circulation would be supposed, whereas increases in abdominal subcutaneous fat would discharge lipolysis outputs into the systemic circulation and evade more

indicate results on hepatic metabolism such as glucose output, lipid synthesis, and emission of prothrombotic proteins such as fibrinogen and plasminogen activator inhibitor 1 (Aubert et al., 2003).

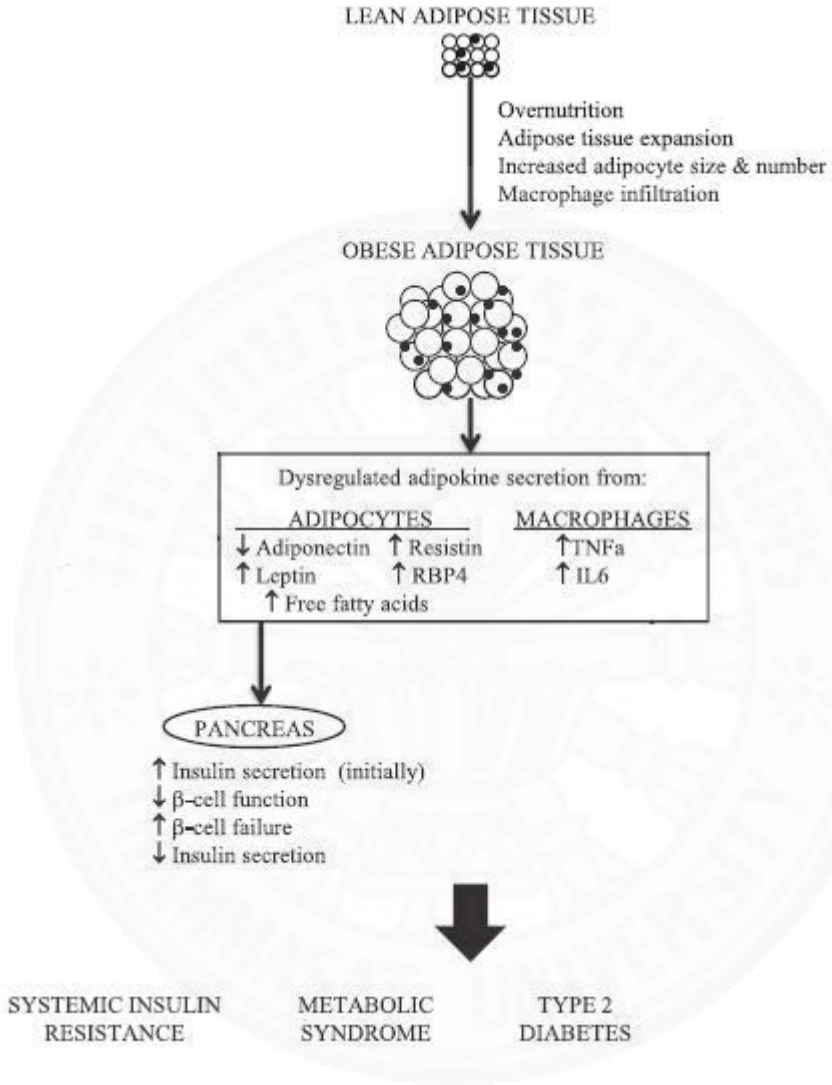


Figure 2.1 The potential pathogenesis of obesity and obesity-related complications (Harwood et al., 2012)

2.2 Metabolic syndrome

2.2.1 Definition and criteria of metabolic syndrome

In recent years, several controversies have involved the definition, criteria, pathogenesis and healthy management of patients with metabolic syndrome, but the

most commonly used defining criteria at the present are from the WHO, the National Cholesterol Education Programme Adult Treatment Panel III (NCEP III), International Diabetes Federation (IDF) (2005) and the European group for the study of Insulin Resistance (EGIR). Although each meaning occupies common features, there are several parameters that differ. However, the diagnostic profiles of each organization able to indicate the conceptual meaning of metabolic syndrome. Metabolic syndrome known also as the insulin resistance syndrome and syndrome X represents a grouping of interrelated metabolic and cardiovascular risk factors (cardiometabolic risk factors) including obesity (especially abdominal obesity), abnormal glucose homeostasis (insulin resistance, glucose intolerance and high plasma glucose), dyslipidaemia [hypertriglyceridaemia, increased plasma low-density lipoprotein-cholesterol (LDL-C) and decreased plasma high-density lipoprotein-cholesterol-cholesterol (HDL-C)] and hypertension (Table 2.1). This combination of danger components is combined to an elevated threat of cardiovascular disease and diabetes mellitus and more recently has also been linked with different medicinal questions such as non¬alcoholic fatty liver disease, cancer and polycystic ovarian syndrome (Bruce & Byrne, 2009; Scaglione, Chiara, Cariello & Licata, 2010).

The concept of Syndrome X, first introduced in the Banting Lecture 1988 (Reaven, 1988), included individuals that were insulin resistant, compensatory hyperinsulinemic, glucose intolerant and exhibited increased plasma triglycerides and decreased high density lipoprotein cholesterol (HDL-C). The name Syndrome X originates from the algebraic term, the letter X which equals the unknown because the association between insulin resistance and the combination with its risk factors for cardio-vascular diseases (CVD) was unknown (Reaven, 2005). The term Syndrome X has been replaced by insulin resistance syndrome which describes an array of abnormal and clinical syndromes associated with insulin resistance (Figure 2.2) (Reaven, 2005).

Table 2.1

Definition and criteria of metabolic syndrome (table was modified from Huang, 2009)

	NCEP ATP III (2005 revision)	WHO (1999)	EGIR (1999)	IDF (2005)
Absolutely required	None	Insulin resistance[3] (IGT, IFG, T2DM, or other evidence of IR)	Hyperinsulinemia' (plasma insulin >75th percentile)	Central obesity: waist circumference[11]>94 cm (M) or >80 cm (F)
Criteria	Any three of five criteria below	Insulin resistance or diabetes, plus two of five criteria below	Hyperinsulinemia, plus two of four criteria below	Obesity, plus two of four criteria below
Obesity	Waist circumference >40 inches (M) or >35 inches (F)	Waist/hip ratio >0.90 (M) or >0.85 (F), or BMI> 30 kg/m2	Waist circumference >94 cm (M) or >80 cm (F)	Central obesity already required
Hyperglycemia	Fasting glucose >100 mg/dl or Rx	Insulin resistance already required	Insulin resistance already required	Fasting glucose >100 mg/dl
Dyslipidemia	TG >150 mg/dl or Rx	TG >150 mg/dl, or HDL-C <35 mg/dl (M) or <39 mg/dl **(F)**	TG >177 mg/dl or HDL-C <39 mg/dl	TG >150 mg/dl or Rx
Dyslipidemia (second, separate criteria)	HDL cholesterol <40 mg/dl (M) or <50 mg/dl (F), or Rx			HDL cholesterol <40 mg/dl (M) or <50 mg/dl (F), or Rx
Hypertension	>130 mmHg systolic or >85 mmHg diastolic, or Rx	>140/90 mmHg	>140/90 mmHg or Rx	>130 mmHg systolic or >85 mmHg diastolic, or Rx
Other criteria		Microalbuminuria[13]		

[a] IGT, impaired glucose tolerance; IFG, impaired fasting glucose; T2DM, type 2 diabetes; IR, insulin resistance; other evidence includes euglycemic clamp studies. [b] Urinary albumin excretion >20|xg/min oralbumin-to-creatinine ratio >30 mg/g. [c] Reliable only in patients without T2DM.

[d] Criteria for central obesity (waist circumference) are specific for each population; values given are for European men and women.

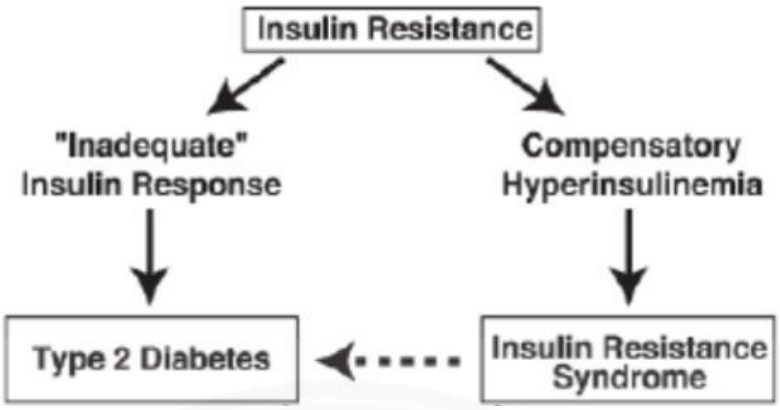


Figure 2.2 Schematic diagram depicting the manifestations of the insulin resistance syndrome (adapted from Reaven, 2005)

The metabolic syndrome comprises a cluster of metabolic disorders which includes increased body mass, abdominal obesity, insulin resistance, hyperglycemia, dyslipidaemia and hypertension (Pittas et al., 2004; Després and Lemieux, 2006; Fulop et al., 2006; Azevedo et al., 2009). These are all threat agents for the generation of type 2 diabetes and CVD and are all principal causes of morbidity and death (Azevedo et al., 2009).

Abdominal obesity is the major determinant and most prevalent manifestation of the metabolic syndrome (Després and Lemieux, 2006; Fulop et al., 2006; Monteiro and Azevedo, 2010). The increase in abdominal obesity leads to adipocyte hypertrophy which causes these cells to rupture and evoke an inflammatory response (Fulop et al., 2006; Monteiro and Azevedo, 2010) which results in a chronic low-grade inflammation (Boden, 2006). Inflammation is driven by adipokines and cytokines (Figure 2.3) secreted by these hypertrophic, adipocytes and macrophages respectively (Fulop et al., 2006).

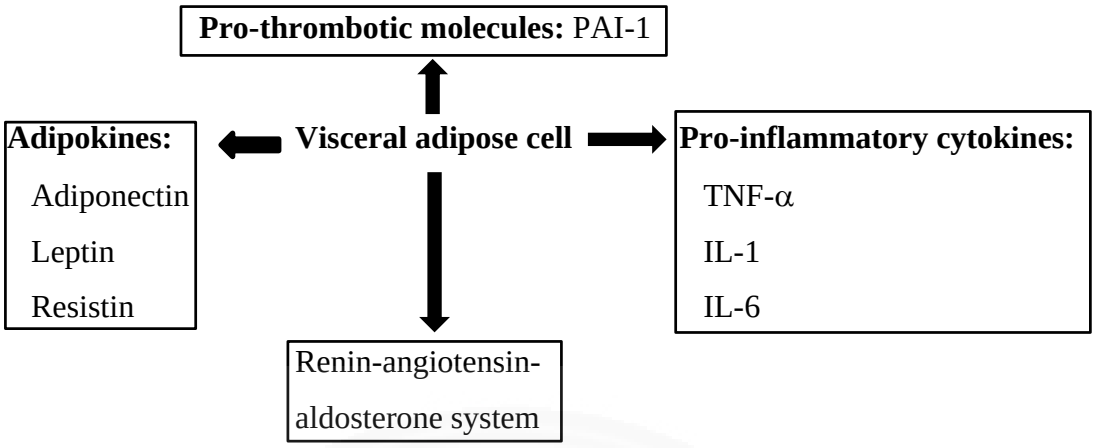


Figure 2.3 Obesity mediated inflammation response (Fulop et al., 2006)

Excess fat deposition in other vital organs, such as the liver, has severe consequences on insulin resistance (Monteiro and Azevedo, 2010). The anatomic distribution of adipose tissue potentially has a major effect on insulin sensitivity. The accumulation of abdominal visceral fat correlates strongly with the generation of insulin resistance (Fulop et al., 2006). In contrast, subcutaneous adipose tissue, which has a large fat storage capacity, acts as a reservoir and has a protective effect against the development of metabolic syndrome (Monteiro and Azevedo, 2010). Viscerally obese individuals represent a sub-group with severe insulin resistance. Insulin resistant, visceral adipocytes cause increased release of FFA from the visceral tissue to the liver resulting in lipoprotein and lipid metabolism disturbances (Fulop et al., 2006). This leads to liver insulin resistance, glucose intolerance, increased triglycerides and LDL-cholesterol which are common features of viscerally obese individuals. In addition to its fat storage function, adipose tissue can be regarded as an endocrine organ due to its ability to release adipocyte-specific factors such as adipokines (Galic et al., 2010). In addition to adipokines, the visceral adipocytes and or infiltrating macrophages secrete pro-inflammatory cytokines of which tumor necrosis factor α (TNF-α) is the most influential. TNF-α modulates the expression of leptin and interleukin- 6 (IL-6) while suppressing adiponectin thereby linking visceral obesity with insulin resistance and metabolic syndrome (Fulop et al., 2006).

2.2.2 Pathogenesis of metabolic syndrome

2.2.2.1 Glucose-stimulated insulin secretion (GSIS)

Glucose is principal stimulus for insulin release from the pancreatic β-cell. Decrease in GSIS *in vivo* has been explained in rats applied the state-of-the-art hyperglycemic hold (van der Heide, Remakers & Smidt, 2006). When the insulin secretory fails are enhance over an increased want for insulin, impaired glucose homeostasis, glucose intolerance, and diabetes can result. Insulin is the greater hormone in adjusting glucose homeostasis. The discharge of insulin from β-cells is a complicated procedure associating the consolidation of many stimuli, for example nutrients, hormones, neurotransmitters, and medicines, but the primitive stimulus for insulin secretion is circulating glucose. The deminish of GSIS is one of the symbols of type 2 diabetes (Del Guerra, Lupi & Marselli, 2005). It is broadly received that there are four influential proceeding associated in GSIS (Figure 2.4);

(1) Glucose is carried into β-cells throughout the translocation of the glucose transporters (GLUTs), specially glucose transporter-2 (GLUT-2), as shown in Figure 2.4.

(2) Formation of ATP via the oxidation of glucose. Later uptake into β-cells, glucose suffers oxidation and finally creates ATP in cytosol and mitochondria through the citric acid cycle moreover implied as the tricarboxylic acid (TCA) cycle, or the Krebs cycle (Figure 2.4).

(3) Estimation of ATP/ADP causes attached of cell-surface ATP-sensitive K^+ (K_{ATP}) channels, preceding to cell membrane depolarization (Figure 2.4).

(4) Ascend in cytosolic Ca^+ activating the exocytosis of insulin granules. The terminal proceeding of insulin secretion is the exocytosis of insulin granules (Figure 2.4). Insulin is reserved in insulin granule, and released by exocytosis.

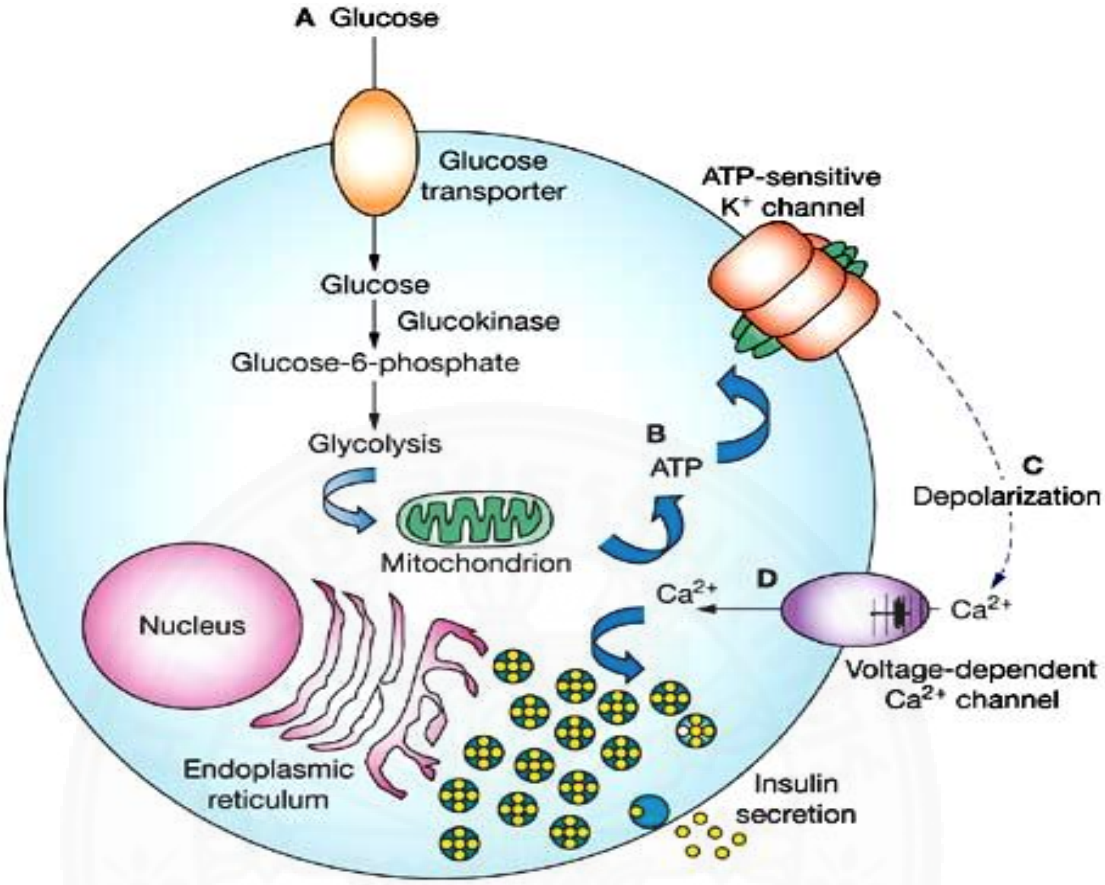


Figure 2.4 The process of glucose-stimulated insulin secretion (GSIS) (León & Stanley, 2007)

2.2.2.2 Insulin resistance and hyperglycemia

Insulin resistance is described as the inability of mark organs, liver, muscle and fat tissues, to retort effectively to insulin stimulation (Pittas et al., 2004; Chakraborty, 2006). Therefore during the development of insulin resistance the body compensates by increasing insulin secretion (Pittas, Joseph, & Greenberg, 2004). To compensate for the forever increasing require for insulin, pancreatic β-cell mass increases exclaim by β-cell hypertrophy or β-cell hyperplasia (Weir and Bonner-Weir, 2007; Fujitani et al., 2010). The symmetry between islet β-cell hypertrophy, β-cell proliferation and β-cell apoptosis directly dictates β-cell mass (Weir, Laybutt, Kaneto, Bonner-Weir, & Sharma, 2001). The magnitude of the working β-cell mass to increase in reply to insulin resistance is critical to anticipate the generation of type 2 diabetes (Weir & Bonner-Weir, 2007). A basic manner of the pathogenesis of diabetes

is the abortion of the pancreatic β-cells to constitute and secrete adequate quantity of insulin to sustain normoglycemia (Weir et al., 2001).

A high-fat diet (with a high component of saturated fat) and specific FFA (mainly saturated FFA) both reduce the expression of the glucose sensing genes, the glucose transporter, GLUT-2 and the glycolytic enzyme, GK. Both high-fat diet and FFA inhibit insulin biosynthesis, reduce insulin gene expression and decrease insulin content. Furthermore, a high-fat diet and long-term exposure to FFA impairs GSIS and reduces circulating insulin concentrations (Figure 2.5).

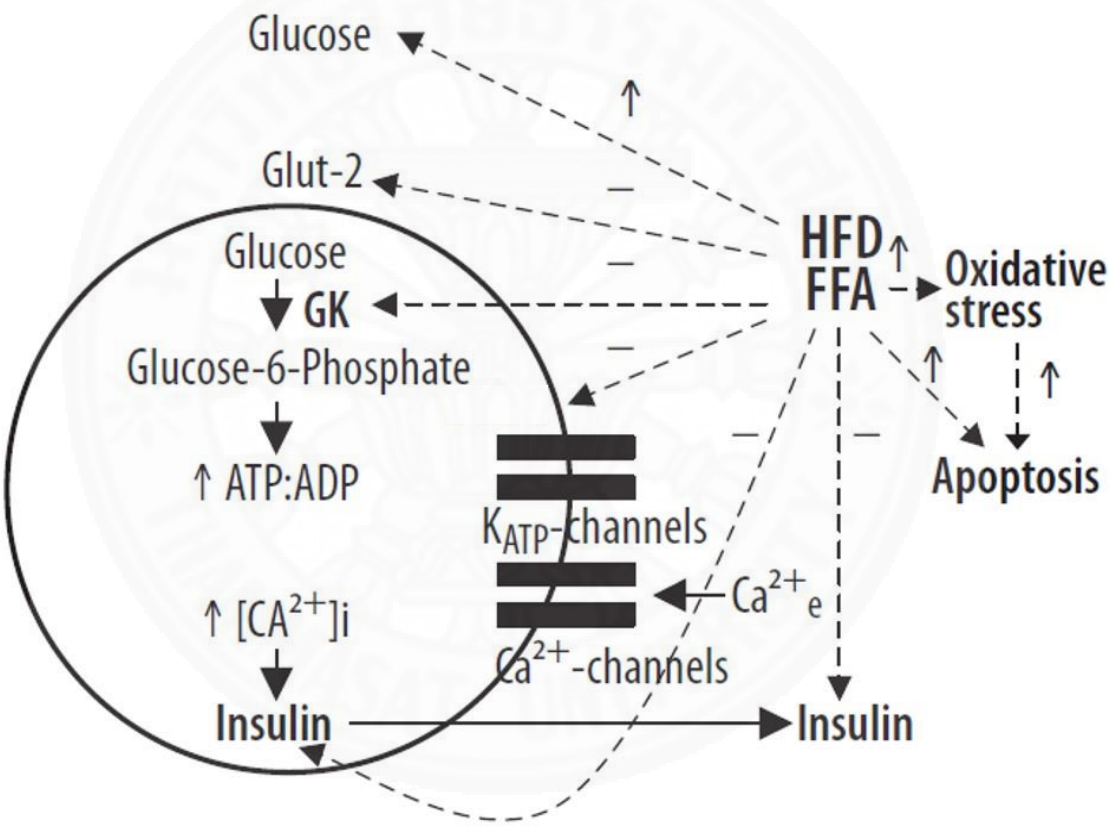


Figure 2.5 High-fat diet regulation of glucose sensing and glucose-stimulated insulin secretion in the β-cell (Cerf, 2007).

2.2.2.3 Insulin signaling

Insulin is a peptide hormone excreted in response to increased circulating glucose levels by the β-cell in the pancreas (Pessin and Saltiel, 2000; Chakraborty, 2006). Insulin is necessary in adjusting carbohydrate, lipid and protein metabolism and the repair of whole-body glucose homeostasis (Pessin and Saltiel,

2000; Chakraborty, 2006; Sesti, 2006). Insulin induces increased lipid synthesis in the liver and fat cells and decreases lipolysis from triglycerides in fat and muscle tissue (Chakraborty, 2006).

IRS proteins compose of four constituents (IRS-1, IRS-2, IRS-3 and IRS-4), play as an interface among insulin and downstream signaling molecules, phosphatidylinositol-3'-kinase (PI3K) (Lingohr et al., 2002; Rhodes & White, 2002). PI3K then phosphorylates the cell membrane phospholipid, phosphatidylinositol (4,5)-biphosphate (PIP_2), converting it to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) (Auger, Serunian, Soltoff, Libby & Cantley, 1989). PIP_3 activating Akt through PDK. Akt in turn positively regulates β-cell mass by activating the cell cycle regulators Cyclin D1 and Cyclin-dependent kinase 4 (Cdk4) as well as regulating several anti-apoptotic genes (Wrede, Dickson, Lingohr, Briaud & Rhodes, 2002). Insulin signaling also stimulates β-cell replication and inhibits apoptosis via IRS-mediated mobilization of the mitogen-activated protein kinase (MAPK) path through growth receptor bound factor 2 (Grb2) (Skolnik et al., 1993). The Src-homology 2 (SH2)-containing adaptor protein (SHC) is bound to the insulin receptor and is activated upon insulin binding. SHC in turn complexes with and activates Grb2 and the guanine nucleotide convert factor Son of Sevenless (SOS), resulting in Ras phosphorylation and activation (Paez-Espinosa et al., 1998). Activated Ras then phosphorylates and activates MAPK, which in turn upregulation of β-cell proliferative and downregulation of β-cell apoptosis.

Insulin-stimulated glucose uptake in muscle and fat is started by the hitching of insulin to the insulin receptor (α-subunit) on the cell surface, resulting in autophosphorylation of multiple tyrosine remainders of the insulin receptor β-subunit in the cytoplasm (Figure 2.6). This results in tyrosine phosphorylation of insulin receptor substrates (IRS-1 and IRS-2). Phosphorylation of the IRS proteins facilitates the binding of Src homology 2 (SH2) domain (p85 subunit) of phosphoinositide 3-kinase (PI3K). Activation of PI3K initiates the PI3K/Akt pathway which results in translocation of glucose transporter four (GLUT-4) from a cytoplasmic pool to the plasma membrane. Glucose is then actively transported into the cell via GLUT-4 located in the plasma membrane.

The PI3K/Akt pathway amusements a pivotal character in insulin signaling and glucose transport via GLUT-4. In type 2 diabetes, PI3K activity decreases in skeletal muscle providing evidence that deficient insulin signaling could result in impaired GLUT-4 mediated glucose transport and insulin resistance (Choi and Kim, 2010). Similarly Akt in muscle from non-obese type 2 diabetes while Akt2 phosphorylation is impaired from adipocytes in obese type 2 diabetes (Choi and Kim, 2010).

Increased concentration of FFA directly or in combination with inflammatory cytokines can cause several failing in insulin signaling (Griffin et al., 1999; Yu et al., 2002). At IRS1, phosphorylation of serine instead of tyrosine causes IRS protein degradation or IRS protein inhibiting the mobilizing of the PI3K/Akt path (Yu et al., 2002; Sesti, 2006). Therefore, GLUT-4 translocation is inhibited and glucose transport into the cell is attenuated (Sesti, 2006).

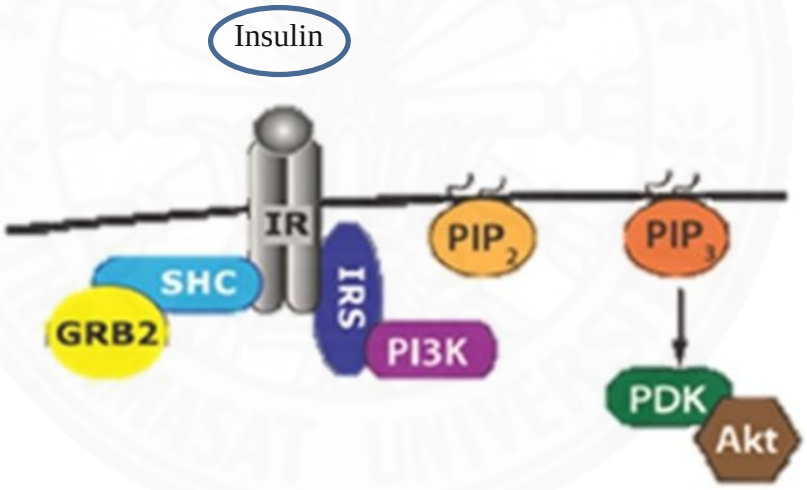


Figure 2.6 Insulin signaling pathway (adapted from Golson, 2010)

Activated Akt is thought to initiate many of the physiological actions of insulin in the adipose tissue, muscle, liver, pancreas, kidney and cardiovascular tissue. This contributes to:

(1) Translocation of glucose transporter to elevate their glucose uptake in adiocyte, myocyte, cardiomyocyte and vascular cells.

(2) Suppression of glucose release from liver and kidney.

(3) Activation of synthesis of glycogen in liver and muscle.

(4) Inhibition of transport of FFA to the bloodstream.

(5) Activation of growth of pancreatic β-cells.

(6) Activation of eNOS activity in the blood vessel.

(7) Activation of cardiac contractility and heart rate.

(8) Induction of anti-apoptotic pathway in the heart.

(Bano, Muniyappa, Montagnani, Koh, & Quon, 2007)

Insulin, created by the pancreatic β-cells, requires peripheral tissue to absorb glucose after feeding. Normally, hitching of insulin to its receptor results in tyrosine phosphorylation of the insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) (Sun et al., 1991), which form complexes with and can phosphorylate associated proteins, resulting in activation of multiple downstream pathways (Backer et al., 1992). For example, IRS is bound to and can activate phosphoinositide 3-kinase (PI3K). PI3K then phosphorylates the cell membrane phospholipid, phosphatidylinositol (4,5)-bisphosphate (PIP2), converting it to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Auger, Serunian, Soltoff, Libby & Cantley, 1989). PIP3 and other targets of TRS in reverse mobilize a variety of downstream purposes with tissue-specific effects. Insulin-responsive tissues include adipose tissue, muscle, liver and β-cell themselves. Obesity modifies how these peripheral tissues respond to insulin, with white adipose tissue, liver and muscle playing major roles in the generation of insulin resistance.

In adipocytes, the Src-homology 2 (SH2)-carrying adaptor protein Shc is bound to the insulin receptor and is activated upon insulin binding. Shc in turn complexes with and activates growth receptor bound factor 2 (Grb2) and the guanine nucleotide transfer agent Son of Sevenless (Sos), resulting in Ras phosphorylation and activation (Paez-Espinosa et al., 1998). Activated Ras then phosphorylates and energizes mitogen-activated protein kinase (MAPK), which in turn upregulates the representation of sterol regulatory element binding protein 1c and 2 (SREBP1c/2). SREBP1c/2 upregulate fatty acid synthase (FAS), thus enhancing the storage of energy as fat and increasing adipose tissue. Additionally, insulin signaling through the IRS normally represses the expression of lipases, which promote lipolysis. Thus, reduction of insulin during fasting states promote the liberation of FFA into the blood stream; increased stores of fat in the obese mean that more FFA are released during

fasting, elevating circulating FFA level. Increased circulating FFA contribute to insulin resistance by impairing insulin signaling, most likely by increasing oxidative stress and increasing inhibitory serine phosphorylation of IRS1/2. In a corrupt cycle, insulin resistance gives to increased levels of FFA in the fed state, since low levels of insulin signaling normally require the body to release FFA because blood glucose level are low. Adipose tissue also cooperates to insulin resistance by releasing transformed levels of hormones and cytokines that regulate metabolism, such as adiponectin and TNF-α. Adiponectin generally sensitixes the body to insulin and is reduced in reply to obesity; in increment to its character in promoting adiposity, SREBP1c also transactivates adiponectin (Seo et al., 2004).

In muscle, PIP3 recruits 3-phosphoinositide dependent kinase 1/2 (PDK 1/2), atypical protein kinase C (aPKC), and the serine-threonine kinase Akt (besides realized as protein kinase B (PKB)), to the membrane, after which PDK1/2 phosphorylates aPKC and Akt stimulate translocation of the glucose transporter-4 (GLUT-4) to the plasma membrane, which allows increased glucose entry. Impaired insulin signaling in muscle finally results in reduced levels of GLUT-4 at the cell sureface and reduced glucose uptake in response to insulin (Tremblay, Lavigne, Jacques & Marette, 2001)

In hepatocytes, signaling through the insulin pathway normally increases activated Akt and aPKC through PDK1/2. Akt in turn phosphorylates the the transcription factor FoxO1, which induces its translocation from the nucleus to the cytoplasm, thus inhibiting its transcriptional activity. In the nucleus, FoxO1 transactivates the promoters of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, both of which are gluconeogenic enzymes; thus, insulin signaling normally inhibits gluconeogenesis, but impaired insulin signaling leads to abnormal hepatic glucose production and output and increased blood glucose levels. In addition to their other roles in the response to insulin signaling, in the hepatocyte, aPKC activates transcription of SREBP1c, while Akt stimulates processing of SREBP1c, which is required for its activity. As in adipocytes, SREBP1c transactivates FAS and promotes fat storage within the liver, which firmly reduces overall insulin sensitivity; increased fat storage in the liver is likely a contributing event in the evolution of type 2 diabetes (Matsumoto et al., 2003).

In β-cell, increased insulin resistance elevates blood glucose levels, because peripheral tissues no longer take up as much glucose and the liver releases abnormally high amounts of glucose. In response, β-cells produce more insulin, both by secreting more insulin per β-cell and by increasing β-cell mass. B-cell proliferation is the primary method by which β-cell mass increases during obesity, stimulated by increased blood insulin and glucose concentration (Sachdeva & Stoffers, 2009).

As in peripheral tissues, insulin signaling in β-cells conducts to increased levels of PIP3 activating Akt through PDK1/2 (Okada et al., 2007). Akt in turn positively regulates β-cells mass by activating the cell cycle regulators Cyclin D1 and cyclin-dependent kinase 4 (Fatrai et al., 2006) as well as regulating several anti-apoptotic genes (Wrede et al., 2002). Insulin signaling also stimulates β-cell replication and inhibits apoptosis via IRS-mediated activation of the MAPK pathway through Grb2 (Skolnik et al., 1993). Impaired insulin signaling in β-cells leads to increased apoptosis and decreased proliferation (Figure 2.7). Although β-cell mass expansion and increased insulin secretion can compensate for elevated insulin demand during the initial stages of insulin-resistance-induced glucose intolerance, studies in diabetic patients and in rodents suggest that diabetes results when β-cells can no longer proliferate or secrete adequate insulin to balance for insulin resistance.

Impaired insulin signaling in β-cells leads to increased apoptosis and decreased proliferation (Figure 2.7). IRS-2 amusements an importance character in the management of pancreatic β-cell growth (Lingohr et al., 2002; Kubota et al., 2000). This has had harrated to the pathogenesis of type-2 diabetes (Lingohr et al., 2002). In the pre-diabetic state insulin target tissues become resistant to normal circulating insulin concentrations. As a effect there is an increased demand for insulin, and so as to compensate for peripheral insulin resistance, β-cell perform and β-cell mass increase. However, in regard to time and/or increased insulin resistance there comes a point where β-cell mass can no longer compensate and type 2 diabetes then establish (Lingohr et al., 2002)

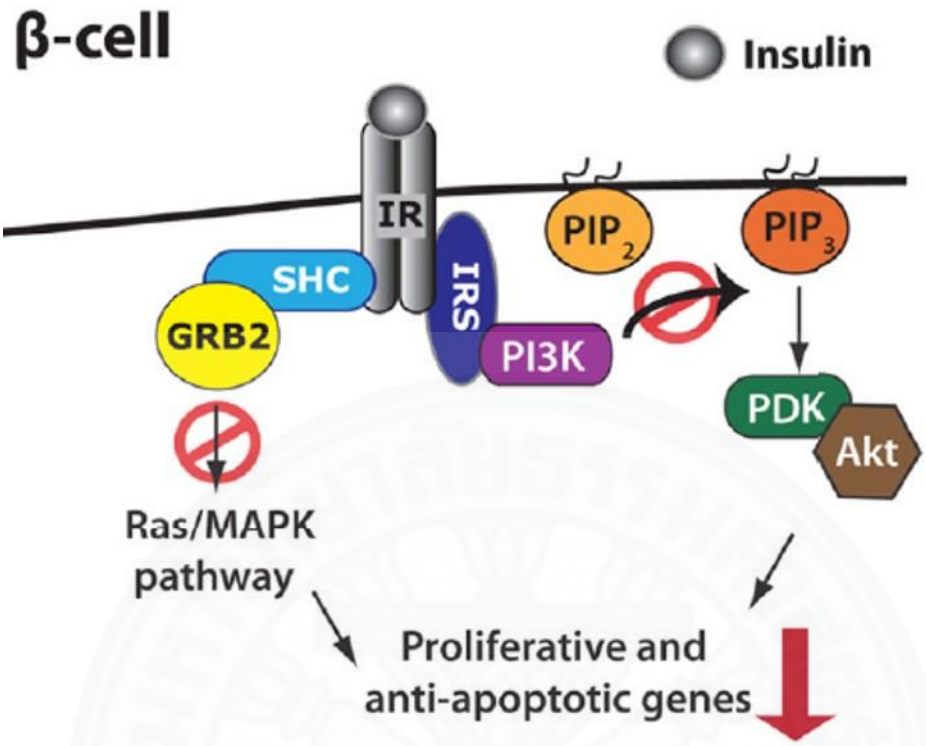


Figure 2.7 The effects of obesity-induced impaired insulin signaling. Red arrows indicate the ultimate effect of impaired insulin signaling in a given cell type, while red circles indicate initiating events that do not occur because of impaired insulin signaling. In β-cells, impaired insulin signaling results in decreased expression of proliferative and anti-apoptotic genes, contributing to reduced β-cell mass (Golson et al., 2010).

2.2.3 Lipotoxicity

The lipotoxic hypothesis was first described by Urger in 1995. In animal and human studies, the progression of obesity is not only connected with elevated lipid accumulation in classically adipose tissue, but can also be deposited within (intracellularly) and around non-adipose tissue (intercellularly). Excess lipids may lead to the overproduction of lipotoxic intermediates that can mediate many detrimental effects, such as insulin resistance, inflammation, oxidative stress, and apoptosis. This phenomenon, known as lipotoxicity or ectopic fat accumulation or steatosis, may amusement an significant capacity in the pathogenesis of MS and its described diseases. Although the exact procedure of lipotoxicity are not favourably implicit, they are linked to the dysregulation of lipid metabolism. The major

pathogenesis of cardiac lipid accumulation involves (DeFronzo, 2010; Schaffer, 2003):

(1) Excessive energy intake
(2) Increased circulating free FFA or TG
(3) Increased FFA uptake
(4) Impaired fatty acid (FA)oxidation (or enhancing oxidation)
(5) Increased synthesis of lipotoxic metabolites and related metabolites for example acyl-CoA, diacylglycerol (DAG), TG, and ceramide

High circulating FFA or TG levels contribute to augmented FFA supply into non-adipose tissue, leading to intracellular lipid accumulation. Alternatively, lipid overload results from imbalance between FFA uptake and utilization. The consequences of lipotoxicity involved the insulin resistance, inflammation, oxidative stress and lipoapoptotic pathway in various organs (Li, Klett, & Coleman, 2010; Unger, 2005). Evidences from man studies and creature models suggest that excessive lipid accumulation in the heart, blood vessel, liver, skeletal muscle, pancreas, macophage, kidney and brain play an significant role in the pathogenesis of metabolic syndrome and its described diseases (Gastaldelli, A. 2011). Insight into these mechanisms may precede to the generation of more efficient preventions and therapy for lipotxocity-related human disease.

All of the main organs are directly impairment of structure and functions in response to chronic exposure to excess lipotoxic metabolites. However, adipose tissue and liver are the primary organs or driver organs for the initiation of metabolic fuel homeostasis in the setting of over nutrition and obesity. The failure of adipocytes and hepatocytes has adequately excess lipids, resulting in lipids redirection to other organs, especially in cardiovascular tissue (Muoio & Newgard, 2006).

2.3 Pancreas

Pancreas is a digestive organ that produces and secretes digestive fluids containing various digestive enzymes via its exocrine acinar cells and ducts. It contains endocrine cells that appear as discrete cell clusters called islets containing a heterogeneous population of neuroendocrine cells which produce four major

hormones namely insulin (secreted by the β-cells), glucagon (secreted by the α-cells), somatostatin (secreted by the δ-cells) and pancreatic poly-peptide (PP-cells) (Ganong, 1989; Stevens & Lowe, 2005). Insulin and glucagon have important functions in the metabolism of carbohydrates, proteins and fats and plays a major capacity in maintenance of glucose homeostasis (Ganong, 1989).

In 1939 H.P. Himsworth observed that certain patients were more sensitive to the hypoglycemic effect of insulin than others that required higher doses of insulin (Reaven, 2005). These observations were the first reference to the concept of impaired insulin action or insulin resistance (Reaven, 2005) which is a pivotal agent in the generation and pathogenesis of T2DM (WHO, 2006).

2.3.1 Pancreatic β-cell function

The pancreatic β-cell is the key cell in the body that produces and secretes insulin, the only hypoglycaemic hormone. β-cells are located within the islets of Langerhans of the pancreas and represent approximately 80% of the islet mass (which itself represents 2-3% of the total pancreatic mass), the remaining 20% being accounted for by glucagon-secreting α-cells, somatostatin-secreting δ-cells and pancreatic polypeptide-secreting PP cells. Under normal conditions, blood glucose levels are sustained within a very restricted range despite large variations in food intake and energy expenditure. This is accomplished by a very tight regulation of insulin secretion from the β-cell, which continuously adjusts its output in response to metabolic, hormonal and neuronal signals. Among these, glucose is the major regulator of insulin release. The β-cell is implemented with a glucose-sensing devices consisting of the glucose transporter GLUT-2 and the high-Michaelis-Menten kinetic (Km), low-affinity isoform of hexokinase glucokinase, the kinetic properties of which certify that intracellular glucose concentrations rapidly equilibrate with extracellular levels within the physiological and supraphysiological range of glycemia (5.5-16.7 mM) (Henquin, 2000; Henquin, 2009).

2.3.2 Pancreatic β-cell mass

There are three principal factors that closely adjust β-cell mass i.e. proliferation, apoptosis and neogenesis, as shown in Figure 2.8 (Gong & Muzumdar, 2012).A key issue in the implying of the pathogenesis of diabetes is the regulation of β-cell mass (Bouwens & Rooman, 2005). At birth, the number of β-cells present is

mostly created by proliferation and differentiation of pancreatic progenitor cells through neogenesis (Bouwens & Rooman, 2005; de Koning, 2008; Marchetti et al., 2008; Rhodes, 2005). After birth, a ration of cycling β-cells can extend in cell count to balance for an gained insulin demand mainly by β-cell proliferation and to a lesser span by β-cell neogenesis (Bouwens & Rooman, 2005). β-cell proliferation is increased and the rate of apoptosis is low which creates the increased β-cell growth early in life. These growth mechanisms adjust during childhood and adolescence which creates equilibrium of sufficient β-cell mass thought adulthood (Marchetti et al., 2008). With aging, the β-cell mass decreases as the rate of apoptosis outweigh proliferation (Marchetti et al., 2008; Rhodes, 2005). This could explain why the elderly are more prone to develop T2DM (Rhodes, 2005).

The lifespan of β-cells in rodents is estimated at approximately 60 days with approximately 0.5% of adult β-cells undergoing apoptosis (Rhodes, 2005). Normally the resultant loss of β-cells is compensated by β-cell replication (Rhodes, 2005). β-cell failure occurs when the critical β-cell mass declines to such an extent that the remaining β-cells are unable to balance for the insulin requirements (Prentki et al., 2006). The most likely mechanisms involved in early β-cell mass reduction includes mitochondrial dysfunction, oxidative stress, endoplasmic reticulum stress, dysfunctional triglycerides, FFA and glucolipotoxicity (Prentki et al., 2006). The subsequent development of hyperglycemia includes accession procedures binded to glucotoxicity including islet inflammation, O-linked glycosylation and amyloid entrusting that accelerates β-cell apoptosis (Prentki et al., 2006). Amylin (islet amyloid poly-peptide- IAPP) co-secreted with insulin, has been implicated in β-cell failure and is the precursor of amyloid deposition in T2DM (DeFronzo, 2004). Elevated plasma IAPP levels have been associated with T2DM patients, obese glucose intolerant subjects and in animal models of diabetes (DeFronzo, 2004). It is suggested that elevated levels of FFA and amylin hypersecretion as is found in insulin resistance acts synergistically to impair β-cell function and cause β-cell injury (DeFronzo, 2004).

Glucotoxicity is associated with hyperglycemia that causes β-cell desensitization to glucose and increased apoptosis (Marchetti et al., 2008). Clinical evidence has proven that lowered serum glucose concentrations in T2DM patients can

cause an increase in their acute insulin response to glucose (Marchetti et al., 2008). Glucotoxicity is associated with inflammatory cytokines, like interleukin one beta (IL-1β), secreted by human islets, in the appearance of peak glucose concentrations which can mediate β-cell apoptosis (Maedler et al., 2002).

In insulin resistant and T2DM patients, dyslipidemia and resultant lipotoxicity, associated with the accumulation of FFA and their metabolic products have a deleterious effect on β-cells (Maedler et al., 2002). In addition, lipotoxicity alters insulin signaling in the liver and skeletal muscle thereby contributing to whole body insulin resistance and deterioration of glucose tolerance (Galgani et al., 2008). A study inducing glucolipotoxicity in Wistar rats caused insulin resistance at six months but not in two month old rats which suggests that older animals should be used in studies of diet induced insulin resistance and which closely mimic typical T2DM (Fontés et al., 2010).

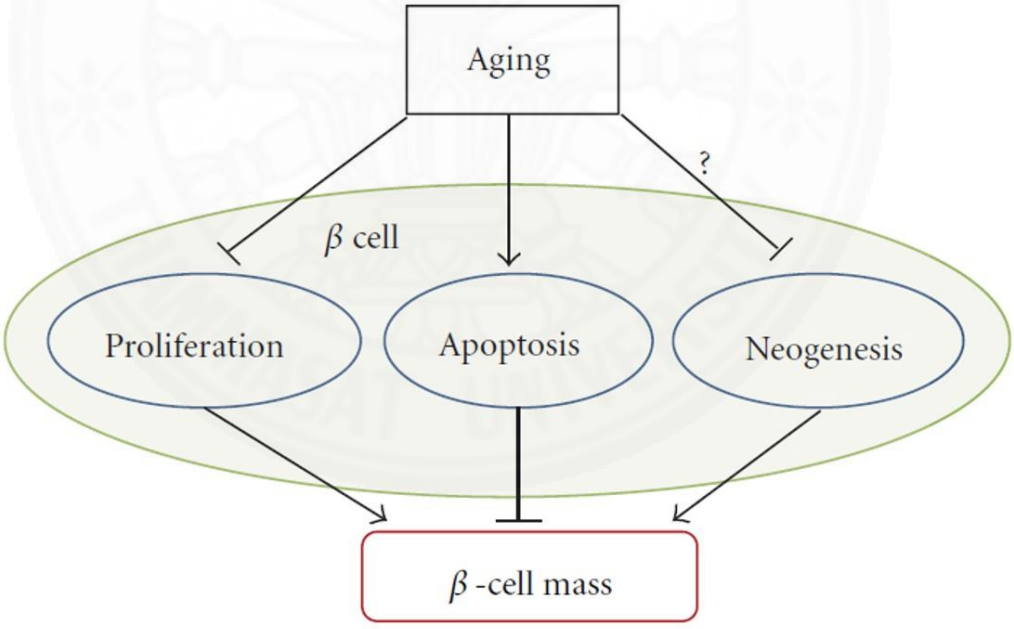


Figure 2.8 Agents that hold β-cell mass. β-cell proliferation, apoptosis and neogenesis are three principal agents that restraint β-cell mass (adapted from Gong & Muzumdar, 2012).

2.3.3 Pancreatic β-cell compensation to insulin resistance

Insulin resistance, a characteristic attribute of the metabolic syndrome, increases the contents on the β-cell to produce more insulin to remunerate for its decreased efficiency. In humans, this is believed to occur via two complementary mechanisms; enhanced insulin secretion and increased β-cell mass.

The initial observation that β-cell definite eradication of the insulin receptor leads to a decrease in β-cell mass and faulty glucose-induced insulin secretion (Kulkarni et al., 1999) provided the first demonstration for a character of insulin signaling in the β-cell in glucose homeostasis. This has been confirmed by subsequent studies, examining the consequences of β-cell specific deletion of genes encoding proteins in the insulin signaling pathway (Kasuga, 2006), such as insulin receptor substrate-2 (IRS-2) (Choudhury et al., 2005; Kubota et al., 2004) and phosphatidyl-inositol-3 kinase (PI3K) (Kaneko et al., 2010).

2.3.4 Pancreatic β-cell decompensation and failure

2.3.4.1 Pancreatic glucolipotoxicity

The metabolic syndrome provides a situation in which dyslipidemia (lipotoxicity) and hyperglycemia (glucotoxicity) are simultaneously present and synergistically alter the function of multiple organs, including the β-cell (Poitout & Robertson, 2008). Accordingly, a large number of *ex vivo* and *in vivo* studies in rats have shown that excessive fatty acids levels, in the appearance of high glucose, have deleterious results on β-cell function and, in some cases, viability.

2.3.4.2 Pancreatic inflammation

Obesity is associated low-grade inflammation. More specifically, expansion of visceral white adipose tissue triggers a systemic inflammatory process which negatively impact glucose homeostasis and create a corrupt cycle contributing to the onset of diabetes (Muoio & Newgard, 2008). At the regular of the β-cell, interleukin-1β (IL-1β) is proposed to amusement a major role both in type 1 and type 2 diabetes (Donath, Storling, Maedler & Mandrup-Poulsen, 2003). Another cytokine proposed to modulate islet function in the metabolic syndrome is IL-6. Circulating regular of IL-6 are increased in obesity and are predictive of type 2 diabetes (Spranger et al., 2003; Hoene & Weigert, 2008). Pancreatitis is inflammation in the pancreas. Pancreatic injury happens when the digestive enzymes are activated before they are

discharged into the small intestine and begin encountering the pancreas. There are two types of pancreatitis, acute and chronic pancreatitis.

Persistent activation of NF-κB leads to: loss of differentiated-cell functions by down-regulation of pancreatic duodenal homeobox-1 (Pdx-1), up-adjustment of inducible nitric oxide synthase (iNOS) and excessive NO production, up-adjustment of chemokines such as monocyte chemoattractant protein-1 (MCP-1), and down-regulation of the CA^{2+} pump sarcoendoplasmic reticulum CA^{2+} ATPase type 2b (SERCA-2b) (Kharroubi, 2004). Transient activation of NF-κB may be beneficial to insulin secretion from pancreatic islets at the early stage of cytokine stress (Wang, 2010).

In contrast to persistent activation, transient activation of NF-κB may be beneficial to insulin secretion from pancreatic islets at the early stage of cytokine stress. It is possible that NF-κB may be involved in a compensatory mechanism that develops in β-cells during the loss of insulin sensitivity, NF-κB has been shown to be significant for the expression of GLUT-2, which contributes to glucose-stimulated insulin secretion by β-cells. Inhibition of this transcription factor may have deleterious effects leading to the generation of insulin resistance and T2DM.

2.3.4.3 Pancreatic senescence

Cellular senescence is an significant functioning for anticipating the proliferation of latent cancer cells. Currently, although, it has become obvious that this procedure associates more than a common rest of cell growth. In accession to stopping tumor genesis, cellular senescence might also assist tissue repair and fuel inflammation combined with aging and cancer furtherance. Thus, cellular senescence might cooperate in four complicated biological procedures (tumor suppression, tumor promotion, aging, and tissue repair), certain of which have clearly obstructing effects. The question now is to imply the senescence answer favourably sufficient to control its advantages while stopping its distress.

During the pathogenesis of T2DM, proliferation of β-cells is a compensatory process. As β-cells have a ended replication promising, this compensative proliferation might expedited cellular senescence and lead to diabetes. Senescence cells are not resting or finally differentiated cells, though the characteristic is not always sincere. No marker or sign of senescence determined thus

distant is absolutely specific to the senescent state. Nonetheless, senescent cells exhibit various phenotypes, which, in describe, define the senescent state (Figure 2.9). Silent features of senescent cells are;

(1) The senescence growth inhibit is crucially stable and cannot be overturned by known physiological stimuli. Nevertheless, some senescent cells that do not reveal the CDKi p16INK4a can continue development later genetic interferences that inactivate the p53 tumor suppressor (Beauséjour et al., 2003).

(2) Senescent cells gain in mass, occasionally extending more than twofold relevant to the mass of nonsenescent counter-parts (Hayflick, 1965).

(3) Senescent cells reveal a senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995), which partly considers the increase in lysosomal mass (Lee et al., 2006).

(4) Maximal senescent cells reveal p16INK4a, which is not normally revealed by quiescent or terminally differentiated cells (Stein et al., 1999). In some cells, p16INK4a, by mobilizing the pRB tumor suppressor, causes construction of senescence-associated heterochromatin foci (SAHF), which silence pro-proliferative genes (Narita et al., 2003).

(5) Cells that senescence with persevering DNA-damage reply signaling protect insistent nuclear foci, termed DNA segments with chromatin changes reinforce senescence (DNA-SCARS) (Rodier et al., 2009).

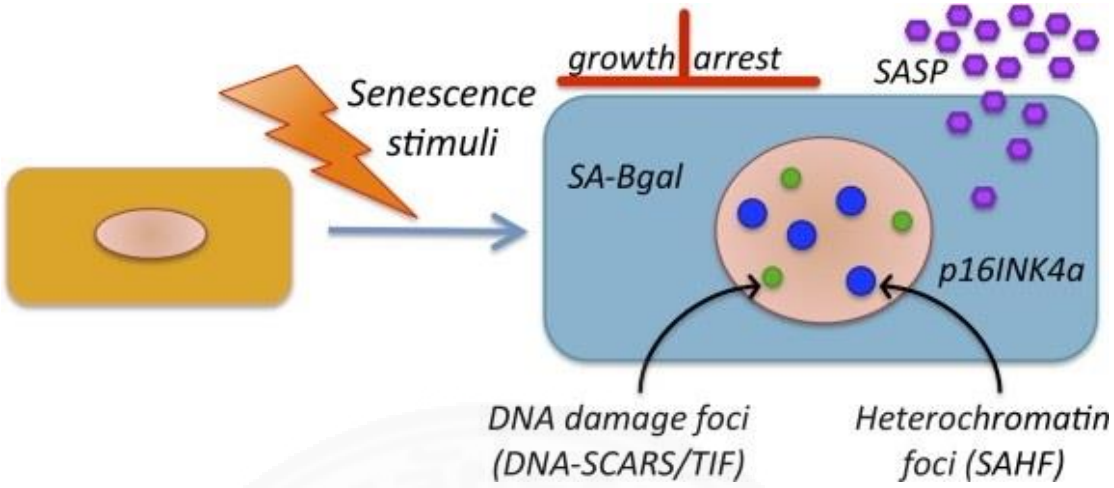


Figure 2.9 Signs of senescent cells (Rodier & Campisi, 2011).

The senescence development stay is crucial stable and cannot be revoked by recognized physiological stimuli. Senescent cells increase in mass, occasionally extending more than twofold relevant to the mass of nonsenescent complements (Hayflick, 1965). Senescent cells reveal a senescence-associated beta-galactosidase (SA-β-gal) (Dimri et al., 1995), which partly considers the increase in lysosomal mass (Lee et al., 2006).

2.4 Metformin

Metformin (dimethylbiguanide) is widely used for the therapy of T2DM. It is an insulin-sensitizing and antihyperglycemic agent applied in the healing of non-insulin-dependent diabetes mellitus (NIDDM). It has been the anchor of therapy of diabetes mellitus for many years, the exact mechanism of metformin is unknown, but one of its suggested actions is increased peripheral glucose disposal at lower insulin concentrations (Prager, Schernthaner & Graf, 1988; Nosadini et al., 1987; Groop et al., 1989). It has been shown that metformin cure of obese adults with type 2 diabetes effects in weight loss and ameliorate glucose tolerance and lipid profiles (Hermann & Melander, 1992; Vigneri & Goldfine, 1987; Bailey, 1992; Abbasi, Carantoni, Chen & Reaven, 1998). Furthermore, the use of metformin in nondiabetic obese adults has been demonstrated to inducement reduced diet intake and weight detriment with reduction in fasting plasma glucose, cholesterol and insulin concentration (Fontbonne et al., 1996; Lee & Morley, 1998; Diamanti-Kandarakis, Kouli, Tsianateli & Bergiele,

1998). On the other hand, short-term metformin use in women with polycystic ovary syndrome and insulin resistance has been shown to develop insulin sensitivity without a significant effect on body weight (Diamanti-Kandarakis, Kouli, Tsianateli & Bergiele, 1998; Morin-Papunen, Koivunen, Ruokonen & Martikainen, 1998). A three-month trial of metformin (500 mg twice daily), in a group of 7 nondiabetes children ages 9 to 14 years, resulted in weight loss and improved insulin sensitivity compared a control group (Lutjens & Smit, 1976). It has useful effects on circulating glucose and lipids (Musi et al., 2002; Wu et al., 1990). It promotes process of transcription and translocation of GLUT4. It is also proposed to inhibit Acetyl CoA carboxylase activity by working through AMP phosphokinase system. (Zhou et al., 2001). The center of metformin's functioning of achievement is the modification of the energy metabolism of the cell. Metformin employs its prevalent, glucose-lowering result by inhibiting hepatic gluconeogenesis and contrast the action of glucagon (Figure 2.10) (Rena, Pearson & Sakamoto, 2013). The restraint of mitochondrial complex I effects in flawed cAMP and protein kinase A signaling in response to glucagon. Stimulation of 5'-AMP-activated protein kinase, although unnecessary for the glucose-lowering result of metformin, consults insulin sensitivity, mainly by adjusting lipid metabolism (Pernicova & Korbonits, 2014). Metformin's ability to increase fatty acid β-oxidation in adipocytes (Gonzalez-Barroso et al., 2012) and its ability to inhibit hepatic lipogenesis (Bhalla et al., 2012).

Hyperinsulinemia and insulin resistance are usual attributes of obesity in both humans and experimental animal (Kay et al., 2001). There are data on the character of metformin in insulin resistance connected with obesity before the development of T2DM in children by improvement in body mass index (BMI), fasting serum glucose, and insulin and improved lipid profile in patients on metformin remedy for exogenous obesity with insulin resistance (Abbasi et al., 1998; Freemark & Bursey, 2001).

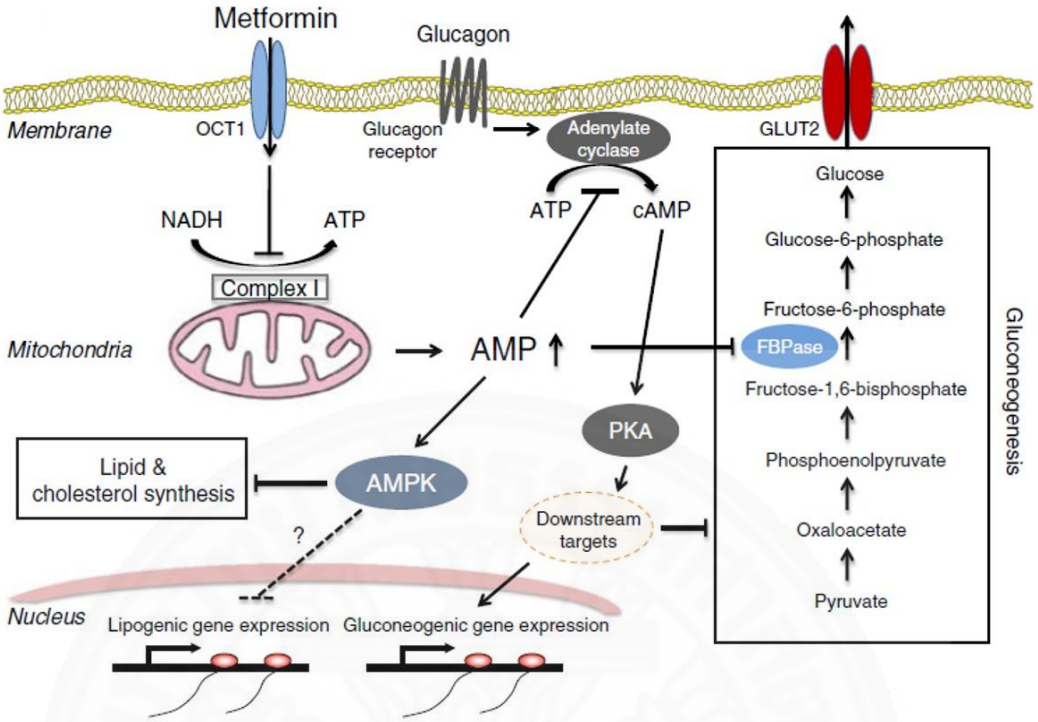


Figure 2.10 Plan draft of the anti-hyperglycaemic achievement of metformin on the liver cell (Rena, Pearson & Sakamoto, 2013)

2.5 Diabetes

Diabetes is defined as a chronic disease involving the β-cells in the pancreatic islets. In diabetes, β-cells cannot produce sufficient insulin or the body cannot efficiently utilize the insulin that is being produced (Zimmett, 2001; WHO, 2009). Diabetes is also associated with micro-vascular and macro-vascular complications which generally reduces quality of life, life expectancy and increases morbidity (Roglic et al., 2006; Zimmet et al., 2001). Uncontrolled diabetes is characterized by hyperglycemia that over time is detrimental to many body systems (WHO, 2009). The recent gain in the occurrence of diabetes is due to many factors including urbanization, diet, obesity and a sedentary lifestyle (Wild et al., 2004; Zimmett et al., 2001).

According to the WHO more than 220 million people world-wide have diabetes and an evaluated 1.1 million people decrease from diabetes in 2005.

Projections indicate that deaths attributed to diabetes will double from 2005 to 2030 (WHO, 2009).

The two principal kinds of diabetes are type 1 diabetes and T2DM. Type 1 diabetes is an auto-immune illness that is characterized by destruction of pancreatic β-cells and leads to deficient insulin secretion. Type 1 diabetes patients require exogenous insulin administration to control hyperglycemia (WHO, 2009; Zimmett et al., 2001). Type 2 diabetes is connected with obesity and sedentary lifestyle and accounts for 90% of people with diabetes (Zimmett et al., 2001; WHO, 2009). According to a review by DeFronzo, 2004, the pathophysiology of T2DM starts with normal glucose tolerance, insulin resistance, compensative hyperinsulinemia, with a furtherance to impaired glucose intolerance and T2DM. The primary defects responsible for the progression to T2DM is impaired insulin secretion by the β-cells, increased glucose production by the liver and decreased utilization of glucose by peripheral tissue such as muscle (DeFronzo, 2010). These phenomenons have been observed in many diverse populations and in animal models such as the rhesus monkey, which closely resembles T2DM in humans (DeFronzo, 2004). Such studies have showed a powerful combination between obesity and the development of T2DM (Freemantle et al., 2008). A decrease in tissue insulin sensitivity with a compensative increase in fasting and glucose-stimulated plasma condensations is the earliest noticeable abnormality that exists before the attack of diabetes (DeFronzo, 2004). When the rate of insulin secretion cannot be maintained any longer due to β-cell failure and loss of β-cell mass, fasting hyperglycemia and glucose intolerance follows which leads to the progression to T2DM (DeFronzo, 2004).

During ingestion of a mixed meal, approximately 50% of glucose is used by the brain, 25% in splanchnic area (liver and gastrointestinal tissues) and the remaining 25% in muscle and to a lesser span in adipose tissue (DeFronzo, 2004). During ingestion of glucose there is an increase in plasma glucose condensations which then in turn excites the liberation of insulin by the pancreatic islets to dispose of excess glucose (DeFronzo, 2004). This causes a temporary state of hyperinsulinemia and hyperglycemia which excites glucose uptake by the splanchnic and muscle tissues (DeFronzo, 2004). A small amount (approximately 4-5%) of glucose is metabolized by adipocytes, although it plays a major part in repair of total

body glucose disposal by the regulation of FFA from stored triglycerides (DeFronzo, 2004). Increased insulin concentrations inhibit lipolysis and cause a deteriorate in plasma FFA concentration, resulting in an increase in muscle glucose uptake. Insulin also suppresses glucose production in the liver (DeFronzo, 2004).

2.6 High-fat diet animal model

Animal models in the study of diabetes are advantageous and offer useful insights into the mechanisms of human diabetes (Srinivasan and Ramarao, 2007). Rodent models fed a high-fat diet have contributed importantly to our comprehending of the pathophysiology of insulin resistance. According to Buettner et al., the first characterization of a high-fat diet used to persuade obesity via nutritional mediation was performed by Masek and Fabry in 1959 (Buettner et al., 2006). Different types of fat in high-fat diets have varying effects on glucose metabolism in Wistar rats. Lard, rich in polyunsaturated fatty acids, and olive oil, affluent in monounsaturated fatty acids, as the main fat components has shown the most evidented representation of obesity and insulin resistance when compared to coconut fat and fish oil (Buettner et al., 2006). These results suggest that the accepted hypothesis that all saturated fatty acids exacerbates insulin resistance, needs further study (Buettner et al., 2006). In the short term (a three to four weeks intervention), high polyunsaturated diet feeding induces insulin resistance without hyperglycemia in Wistar rats (Chalkley et al., 2002). Similarly, Wistar rats fed a safflower oil-based high-fat diet (59% calories as fat) from two months old for a ten months period, developed insulin resistance but not diabetes (Chalkley et al., 2002). It suggested that without a genetic predisposition, the high-fat feeding alone does not result in T2DM (Chalkley et al., 2002). A learn by Krygsman et al., revealed that not only the percentage dietary fat that amusements a character in glucose intolerance and insulin resistance but a relationship between fatty acid composition and dietary fat (Krygsman et al., 2010).

High-fructose corn syrup (HFCS), as sweetener, is normally applied in diet especially drinks, including carbonated sodas (Hofmann and Tschöp, 2009). The use of HFCS has been offered as an influential dietary component that has cooperate to the extensive increase in human obesity discerned in Westernized societies

(Hofmann and Tschöp, 2009). HFCS contains 5% more fructose compared to sucrose ("normal sugar"). Fructose exerts an increased perception of sweetness therefore it is favoured by diet and soft drink producers (Hofmann and Tschöp, 2009).

The addition of fructose to a high-fat diet is normally applied to persuade an animal model of T2DM (Huang et al., 2004). A study done by Huang et al., concluded that a high-fructose diet causes hyperinsulinemia, while a high-fat diet results in deteriorated pancreatic perform of insulin secretion and glucose intolerance. This suggests that high-fructose diet and high-fat diet exerts divergent effects on glucose metabolism in rats (Huang et al., 2004). Relevant differences in metabolism of rats fed fructose-enriched diets showed substantial insulin resistance and hyperinsulinemia in both lean and obese rats, whereas rats fed a glucose-enriched diet led to enhance insulin sensitivity (Suga et al., 2000). The metabolism of these sugars where comprehensively compared in overweight/obese humans and found that visceral adipose output was importantly gained in subjects absorbing fructose (Stanhope and Havel, 2009) when compared to glucose consumption (Stanhope et al., 2009). Fasting plasma glucose and insulin concentrations increased and insulin sensitivity decreased in obedients eating fructose but not those eating glucose (Stanhope et al., 2009). High-fructose diets induce dyslipidemia, decrease insulin sensitivity and increase visceral adiposity (Stanhope et al., 2009).

2.7 Benjakul and metabolic syndrome

Thai traditional medicine (TTM) is a cultural heritage and indigenous wishdom which has helped care the health of Thai people for more than thousand years. Preparations of Thai traditional medicines for illness treatments include several herbs rather than a single herb. Benjakul is one of national lists of essential medicines used in Thai traditional system of medicine. It has been used for balanced health in Thai conventional medicine and also manipulated as an adaptogen and for treatment of dyspepsia in the lists of The National Drug List of Herbal Medicine Products A. D. 2006 (Bureau of Drug Control, 2012). Maha-pigud Solot Benjakul is drug pigud used in 5 dhatues; Patawee dhatu, Apo dhatu, Wayo dhatu, Techo dhatu and Argard dhatu. It has been devided into 5 basis for solve in each dhatu; (1) Maha-pigud Solot

Benjakul solve in Patawee dhatu, (2) Maha-pigud Solot Benjakul solve in Apo dhatu, (3) Maha-pigud Solot Benjakul solve in Wayo dhatu, (4) Maha-pigud Solot Benjakul solve in Techo dhatu and (5) Maha-pigud Solot Benjakul solve in Argard dhatu. Maha-pigud Solot Benjakul solve in Apo dhatu is refer to it can solve T2DM. Maha-pigud Solot Benjakul is composed of five plants (Figure 2.11); *Piper retrofractum* Vahl. fruit 2 parts, *Piper sarmentosum* Roxb. root 16 parts, *Piper interruptum* Opiz. stem 8 parts, *Plumbago indica* Linn. root 6 parts, and *Zingiber mekongense* Gagnep. rhizome 4 parts (part is mean weight of stable dried herb (45 °C)) (Ministry of Education, 1999).

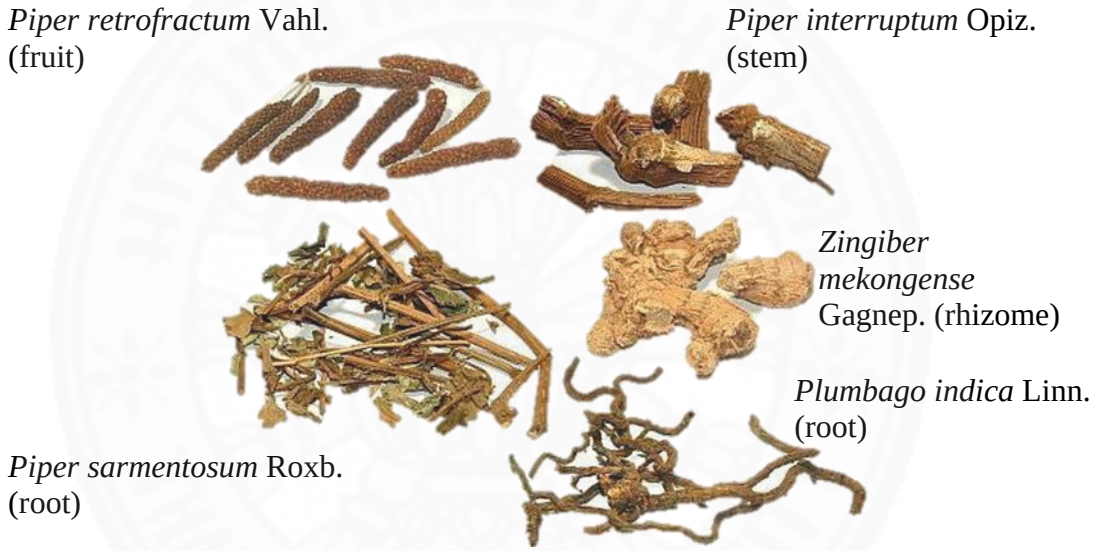


Figure 2.11 Benjakul. Adapted from http://www.samunpri.com/Pictures/PicHerbs/slides/A177.html

In 1999, Itharat and colleagues studied the effect of Benjakul. They found that this therapeutic application is believed to be through balancing “dhatu” (regulating body chemical and physical function) before cancer treatment (Itharat et al., 1999; Sriyakul et al., 2010). Benjakul extract showed no toxicity for body tissue and biomaterial changes when tested by a sub-chronic toxicity method (Chavalittumrong, Attawish, Rungsamon & Chuntapet, 1996).

Piper sarmentosum Roxb. (wild betal leaf bush, Thai name: Chaplu) (Figure 2.12) was a herbal medicine used for controlling the abnormality derived from the existing of excessive water element, a dhatu, in the body. The example of such abnormalities are excess saliva, bloody mouth, dry teeth or too much urinate. It has been broadly used in Asian countries as diet and conventional medication. *Piper sarmentosum* water extract (whole plant and leaves) can reduce blood glucose levels, prevents the degenerative and necrotic changes in the cells (β-cell) of the islets of Langerhans, progression of diabetic nephropathy, and degenerative changes in the cardiovascular tissue in male Wistar rats induced by Streptozotocin. (Peungvicha et al., 1998; Thent et al., 2012; Hussan et al., 2013)

Figure 2.12 *Piper sarmentosum* Roxb. (wild betal leaf bush, Thai name: Chaplu). From http://www.manager.co.th/QOL/ViewNews.aspx?News ID=9510000137171

Piper retrofractum Vahl. (long pepper, Thai name: Di-pli) (Figure 2.13) was a drug used for tonic earth element and controlled abnormal of earth element such as muscle and tendon painful, stress and dried skin. Methanolic extract can reduces cholesterol uptake, synthesis and dietary lipid digestion (Duangjaiac et al., 2011). Piperidine alkaloids such as piperine, pipernonaline and dehydropipernonaline have gastoprotective and hepatoprotective properties. They can induce fat-burning protein expression in 3T3-L1 adipocytes and myocytes (Kim et al., 2011). Piperine can reduces final body weight, aspartate aminotransferase (AST), alanine aminotransferase (ALT), but it can gain liver weight and hepatic triglyceride, cholesterol and FFA meanings in male C57BL/6J mice fed a high-fat diet (Choi et al.,2013).

Figure 2.13 *Piper retrofractum* Vahl. (long pepper, Thai name: Di-pli). From http://www.jiaogulan4u.com/benjakul-samoonpri.html

Piper interruptum Opiz. (pepper wood, Thai name: Sa-kan) (Figure 2.14) was a drug used for tonic wind element and controlled abnormal of wind element such as low or high blood pressure flatulent, dry mouth, emetic or headache. Previous study has shown that the treatment with ethanolic extract of its stem could reduces ear and paw edema and prevents transudative burden and granuloma wet weight in cotton pellet-induced granuloma construction male Sprague Dawley rats (Sireeratawong et al., 2012)

Figure 2.14 *Piper interruptum* Opiz. (pepper wood, Thai name: Sa-kan). From http://www.jiaogulan4u.com/benjakul-samoonpri.html

Plumbago indica Linn. (rose-color lead wood, Thai name: Chettamun-phloeng-daeng) (Figure 2.15) was a drug used for tonic fire element and controlled abnormal of fire element such as dried cough, lower body temperature, beriberi and anorexia. Methanol extracts of roots have steroids, tannins, glycosides, phenols, flavonoids and saponin that have antioxidant activity (Eldhose et al., 2013). Plumbagin in *Plumbago indica* Linn. have anti-inflammatory and analgesic effect through inhibition of NF-κB signaling pathway (Luo et al., 2010).

Figure 2.15 *Plumbago indica* Linn. (rose-color lead wood, Thai name: Chettamun-phloeng-daeng). From http://www.manager.co.th/QOL/ViewNews.aspx?News ID=9510000137171

Zingiber mekongense Gagnep. (ginger, Thai name: Khingheang) (Figure 2.16) was a drug used for tonic air element and controlled abnormal of air element such as blur and tinnitus. Gingerol can cause translocation of GLUT-4 to muscle cell plasma membrane surface and extend glucose uptake in skeletal muscle and adipocyte (Li et al., 2012; Sekiya et al., 2004). Methanolic extract of Z*ingiber officinale* Roscoe. can inhibits cholesterol uptake in Caco-2 cells (Duangjaiac et al., 2011). Ethanolic gingerol extract can reduces plasma total cholesterol, TG and LDL-C levels (Bhandari et al., 1998). Ginger extract diminishes plasma glucose, insulin and TG in chronic fructose consumption-induced kidney damage male Sprague Dawley rats (Yang et al., 2014). Ginger extract can suppresses adipose macrophage-ralated proinflammatory cytokines in fructose-induced adipose tissue insulin resistance rats (Wang et al., 2013). Ginger extract can improves fructose-induced fatty liver and hypertriglyceridemia in rats (Gao et al., 2012).

Figure 2.16 *Zingiber mekongense* Gagnep. (ginger, Thai name: Khingheang). From http://www.jiaogulan4u.com/benjakul-samoonpri.html

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Material

3.1.1 Instruments

1. Electrical balance (Mettler-Toledo, Bangkok, Thailand)
   Electrical balance (Precisa Instrument, Dietikon, Switzerland)
2. Autopipettes (Rainin Instrument LLC, Columbus, OH, USA)
3. Hotplate and stirrer (Sterillin, Charles de Gaulle, France)
4. Hot air oven (ULM 600) (Memmert, Bavaria, Germany)
5. Autoclaver (Hirayama, Tokyo, Japan)
6. Centrifuge machine (Beckman coulter, Brea, CA, USA)
   Centrifuge machine (Sorvall RC6 –PLUS) (Thermo Electron Corporation, Beverly, MA, USA)
   Refrigerated centrifuge machine (Hettich Zentrifugen, Lauenau, Germany)
7. Microcentrifuge machine (Denville Scientific, Holliston, MA, USA)
   Microcentrifuge machine (Bertec Enterprise, New Taipei City, Taiwan)
8. Spectrophotometer (Shimadzu, Otsushi Shiga, Japan)
9. PowerWave XS Microplate reader (BioTek, Winooski, VT, USA)
10. Digital Dry Bath Incubator (Boekel Scientific, Feasterville, PA, USA)
11. Incubator shaker (JP Selecta, Barcelona, Spain)
12. Orbital Shaker OS-20 (Biosan, Riga, Latvia)
13. Freeze dryer (Lyophilization Systems Inc., Kingston, NY, USA)
14. ACCU-CHEK performa (Roche Diagnostics, Zurich, Switzerland)
15. Sonicator (Elma, Singen, Germany)
16. Homoginizer (Eberbach corporation, Ann Arbor, MI, USA)
17. Eclipse Ci-L microscope equipped to a DS-Fi2 microscope camera (Nikon, Tokyo, Japan)
18. Water bath (Memmert, Bavaria, Germany)

19. NanoDrop 2000 spectrophotometer (Thermo Scientific, West Palm Beach, FL, USA)
20. MyCycler thermal cycler (Bio-Rad, Alfred Nobel Drive Hercules, CA, USA)
21. StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA)
22. MicroAmp Optical 96-Well Reaction Plate and MicroAmp Optical Adhesive Film (Applied Biosystems, Waltham, MA USA)
23. Olympus BX60 light microscope equipped with a digital camera and interfaced with NIH Image 1.63 software (Nikon, Tokyo, Japan)
24. Mini PAP Pen (Sigma-Aldrich, St. Louis, MO, USA)
25. GraphPad (GraphPad Software inc., San Diego, CA, USA)
26. Leica TP 1020 (Leica Microsystems, Nussloch, Germany)
27. Leica RM 2125 RM (Leica Microsystems, Nussloch, Germany)
28. Olympus CX31 microsco equipped to a Olympus DP20 microscope camera (Olympus, Tokyo, Japan)

3.1.2 Chemicals

1. Sodium chloride (NaCl) (Amresco, Solon, OH, USA)
2. Phosphate buffered saline (PBS) tablets (Amresco, Solon, OH, USA)
3. Metformin (Abbot laboratories , Bangkok, Thailand)
4. D-glucose ($C_6H_{12}O_6$) (Amersham Biosciences, Piscataway, NJ, USA)
5. Folin-Ciocalteu pherol reagent (Fluka, Newport News, VA, USA)
6. Sodium carbonate (Na_2CO_3) (Alpha, Bangalore, India)
7. Gallic acid (Sarstedt, Nümbrecht, Germany)
8. Ethanol absolute (Merck, Darmstadt, Germany)
9. Chloroform and analytical reagent (RCI labscan, Bangkok, Thailand)
10. Methanol, analytical reagent (Quality control chemical, Auckland, New Zealand
11. Butylate hydroxytoluene (BHT) (Merck, Darmstadt, Germany)
12. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Fluka, Newport News, VA, USA)

13. Pentobarbital sodium (nembutal) (Jagsonpal, New Delhi, India)
14. Formaline (40%) (International Resin, Shanghai, China)
15. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{}^*H_2O$) (Merck, Darmstadt, Germany)
16. Di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Quality control chemical, Auckland, New Zealand)
17. Fluitest Cholesterol reagent (Analyticon Biotechnologies AG, Lichtenfels, Germany)
18. Fluitest TG reagent (Analyticon Biotechnologies AG, Lichtenfels, Germany)
19. Fluitest HDL-C precipitation reagent (Analyticon Biotechnologies AG, Lichtenfels, Germany)
20. Potassium hydroxide (KOH) (Merck, Darmstadt,Germany)
21. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, St. Louis, MO, USA)
22. Glycerol standards (Sigma-Aldrich, St. Louis, MO, USA)
23. Free glycerol reagent (Sigma-Aldrich, St. Louis, MO, USA)
24. RNA*later* RNA stabilization reagent (Qiagen, Hilden, Germany)
25. TRIzol reagent (Invitrogen, Carlsbad, CA, USA)
26. 2-propanol (Sigma-Aldrich, St. Louis, MO, USA)
27. Diethylpyrocarbonate (DEPC)-treated water (Ambion, Austin, TX, USA)
28. High Capacity cDNA Reverse Transcription Kits (Applied Biosystems,Waltham, MA, USA)
29. TaqMan Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA)
30. TaqMan Gene Expression Assay (Applied Biosystems, Waltham, MA, USA)
    - GK (Assay ID Rn00561265_m1)
    - IRS-2 (Assay ID Rn1482270_s1)
    - GLUT-2 (Assay ID Rn00563565_m1)
    - NF-κB p65 (Assay ID Rn01502266_m1)
    - β-actin (Assay ID Rn00667869_m1)
31. Xylene (Sigma-Aldrich, St. Louis, MO, USA)

32. Hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA)
33. H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA)
34. Tween (Sigma-Aldrich, St. Louis, MO, USA)
35. Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA)
36. β-Galactosidase polyclonal antibody (Abnova, Taipei City, Taiwan)
37. HRP Donkey anti-rabbit IgG (BioLegend, San Diego, CA, USA)
38. DAB Chromogen (Diagnostic BioSystems, Pleasanton, CA, USA)
39. Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA)
40. Entellan® (Merck, Darmstadt, Germany)

3.2 Methodology

3.2.1 Preparation of BWE and WWE

BWE contains unequal parts from five plants as follows: 2 parts of long pepper fruit, 16 parts of wild betel leaf bush root, 8 parts of pepper wood stem, 6 parts of rose-color lead wood root, and 4 parts of ginger rhizome. WWE, the single herb extract, contains only the root of one plant: *P. sarmentosum* Roxb. The plant materials were collected from Rayong Province, Thailand (APPENDIX B). The authenticity of the plant materials was verified by comparing them with the specimens deposited at the Herbarium of the Royal Forest Department, Bangkok, Thailand, where herbarium vouchers are maintained. All plant were cleaned with water to remove extraneous material and then sliced and dried at 55 °C. Then they were grounded using an electric grinder to obtain a powder and extracted in a similar way as that practiced by the Thai traditional doctors. The powdered material from Benjakul with water (water:plant = 2:1 part) was decocted at 100 °C until only 1 part of water remained, and then it was filtered. The filtrates were evaporated using a freeze-drying. The yield (% w/w) for extraction was calculated, and the dried extract was kept in the freezer at –20 °C until further use. Dose of BWE was calculated according to the following equation (Reagan-Shaw, Nihal & Ahmad, 2008):

Adult human eat 2 g × 3 times/day (APPENDIX C) = 6 g/day

Benjakul yield = 6.7% $= \frac{6.7 \times 6}{100}$ = 0.4 g/day

Dose = $0.4 \times \frac{37}{6 \times 60}$ = 0.0413 g/kg BW/day

= 41.3 mg/kg BW/day

Dose of WWE was calculated according to the following equation (Reagan-Shaw, Nihal & Ahmad, 2008): Wild betal leaf bush 7 stalks (http://www.rspg.or.th/plants_data/herbs/herbs_27_1.htm) (APPENDIX D)

Wild betal leaf bush 7 stalks (wet weight) = 20 g = 5 g (dry weight)

Wild betal leaf bush yield = 3.3% = $3.3 \times \frac{5}{100}$ = 0.165 g/d

Dose = $0.165 \times \frac{37}{6 \times 60}$ = 0.017 g/kg BW/day

= 17 mg/kg BW/day

3.2.2 Determination of total phenolic compounds

The total phenolic compounds of BWE and WWE were determined using the procedure of previous studied with some modification (Singleton, & Rossi, 1965). Briefly, 70 µL of BWE and WWE solution were pipetted into a test tube. A 525 µL of diluted Folin-Ciocalteu reagent (1:5 with DW) was added to each of the test tubes. This mixture was shaken for one min. 525 µL of 7.5% Na_2CO_3 was added and shaken. The color of mixture was developed for 30 min at room temperature. The absorbance of the mixture was calculated at 760 nm against the reagent blank.

The total phenolic compound in samples was quantified by the calibration curve of reference standard. Using gallic acid as a reference standard, total phenolic mixtures are indicated as mg of gallic acid equivalents (GAE)/g dry weight of BWE and WWE.

3.2.3 Determination of antioxidant activity

Antioxidant activity of BWE and WWE were determined by scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) generated by the chemical method according to protocol by Yu et al., 2002. BWE and WWE were diluted in 70% ethanol, to a final concentration of 10, 50, 100, 250, 500 and 1,000 µg/mL. An aliquot of 500 µL of sample solution was mixed with 500 µL of 6×10^{-5} M DPPH in absolute ethanol. The absorbance of each sample was calculated at 517 nm by a microplate reader after the reaction mixture is authorized to bear for 30 min at room temperature

in dark. Compare DPPH radical scavenging capacity of each sample with BHT. BHT was used as reference standard tested in the same system. All tests were conducted in triplicate assay. The results were expressed as the inhibition percentage determined from the difference in absorbance (A) of DPPH between the control and sample. The result is reported as half maximal effective concentration (EC50). EC50 value was calculated by Prism GraphPad software (GraphPad Software, Inc., USA). A percentage inhibition activity was calculated by using the formula below.

$$\text{Inhibition (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}}) \times 100}{A_{\text{control}}} \qquad A = \text{Absorbance}$$

3.2.4 Animal model and experimental protocol

3.2.4.1 Animal model

All animal manners were pre-confirmed by the Animal Ethics Committee at the Faculty of Medicine, Thammasat University (AE 001/2015) (APPENDIX E). Ninety one male outbred Sprague-Dawley rats are used in this study. Their initial weight about 180-220 g and aged 6-8 weeks old are obtained from National Laboratory Animal Centre, Mahidol University at Salaya, Nakon Pathom, Thailand. All animals are cared and housed individually in stainless steel cages under environmentally regulated room with temperature at 24 ± l °C and 60% humidity on a fixed 12:12-h brightness/sleep-dim/awake period (lights on at 6.00 A.M.).

3.2.4.2 Expeimental design

A diet-induced obesity model is modified from previous reports (Chakhonpunya, Sireeratawong, Komindr, & Lerdvuthisopon, 2011; Kaendee, Sireeratawong, Burawat, Utama-Ang, & Lerdvuthisopon, 2009) and is made. All rats have ad libitum with approach to standard rat chow and tap water for 7 days prior to the start of experiments. After seven days of acclimation period, rats are casually separated into 6 experimental groups (n=10 per group) with a same mean body weight. At the beginning of this experimental period, each of 9 groups is fed ad libitum with food and water and subjected to different treatment for 4 (short term; group 1-6) and 16 weeks (intermediate term; group 7-9) as follows:

Group 1 rats receiving standard chow (Control) for 4 weeks (untreated control for short term obese rat)

Group 2 rats receiving high fat diet-induced short term obesity (HF) for 4 weeks

Group 3 rats receiving high fat diet-induced short term obesity are co-fed with BWE at the low dose of 41.3 mg/kg rat weight (HFB1) for 4 weeks

Group 4 rats receiving high fat diet-induced short term obesity are co-fed with BWE at the 10 folds of low dose 413 mg/kg rat weight (HFB10) for 4 weeks

Group 5 rats receiving high fat diet-induced short term obesity are co-fed with wild betal leaf bush at the dose of 17 mg/kg rat weight (HFW) for 4 weeks

Group 6 rats receiving high fat diet-induced short term obesity are co-fed with metformin at the dose of 19.1 mg/kg rat weight (Kay et al., 2001) (HFM) for 4 weeks

In this study, metformin was suspended in distilled water and dispensed orally in a dose of 9.55 mg/kg BW once daily for three day in two weeks. It was increased to 19.1 mg/kg BW for fifteen day according to modified of Kay et al., 2001. The dosage was calculated for rats using a program provided by Food and Drug Administration, USA. (http://www/fda.gov/cder/cancer/animalframe. htm).

Group 7 rats receiving standard chow (control) for 16 weeks (untreated control for intermediate term obese rat)

Group 8 rats receiving high fat diet-induced intermediate term obesity (HF) for 16 weeks

Group 9 rats receiving high fat diet-induced intermediate term obesity are co-fed with BWE at the low dose of 41.3 mg/kg rat weight (HFB1) for 16 weeks

The co-feeding rats are done daily for 4 and 16 weeks by oral gavage. The untreated control and high-fat diet rats are gavaged with an equal volume of distilled water. Amounts of rat body weight and dietary intake (food and energy intake) are monitored and recorded every consecutive day throughout the study period. All experimental procedures are performed at the same time (9-10 a.m.).

3.2.4.3 Animal diets and feeding

The standard chow is acquired from National Laboratory Animal Centre, Mahidol University (CP, SamutPrakarn, Thailand) and consisted of 13, 55 and 31 percent of energy derived from fat, carbohydrate and protein, respectively. The obesity-induced diet is the diet that modified from Claret et al. in 2004. The obesity-induced diet consists of high percentage of energy derived from fat (65, 23 and 11 percent of energy derived from fat, carbohydrate and protein, respectively) and rich in saturated fatty acid and cholesterol. The high-fat diet pellets are prepared in the kitchen of the Nutrition Unit, Thammasat Hospital, Thammasat University. The ingredients of experimental diets are displayed in table 3.1.

Crude dried of Benjakul is dissolved in distilled water and daily administered though oral route in a dose of 41.3 and 413 mg/kg rat weight/day to a co¬feeding group of rats are fed high-fat diet and given BWE during experimental weeks. This dose is so prepared that a constant volume of 0.1 mL/100 g of body weight will be administered to each rat. The control and high fat diet group of animals receive only distilled water in the same volume and through the same route.

The dosage is calculated for rats using a program provided by Food and Drug Administration, USA (http://www.fda.gov/cder/cancer/animalframe.com).

Table 3.1

Composition of high-fat diet as were estimated by Nutri Survey Program from Nutrition Divisions, Department of Health, Ministry of Public Health

Ingredients	Amount (g)	Carbohydrate (g)	Protein (g)	Fat (g)	Energy (g)
Sugar	58	57.7	-	-	223.5
Standard chow	100	41.8	24	4.5	304
Wheat flour	150	114.5	15.5	1.5	546.5
Pork liver	100	2.4	19.9	4	126
Egg white, hen	33	-	3.3	0.4	16.8
Pork belly	100	2.8	13.9	33.5	368.1
Margarine	200	-	0.8	172.6	1,558.8
Egg yolk, hen	200	4	28.6	60.2	672.1
Total	941	223.2	106	276.7	3,816
% kcal	-	23.39	11.11	65.26	-

Note; 941 g of composition mixture gave a result of 745 g biscuit.

3.2.4.4 Measurement of animal body weight and dietary intake

Body weight and dietary intake (food and energy intakes) of the rats were recorded daily throughout the study period.

(1) Calculation of food intake

The food intake per rat in each day was calculated as follows:

Food intake (g/day) = Initial food weight (g) – Leftover food weight (g)

(2) Calculation of energy intake

The energy intake per each rat per day was calculated as:

Energy intake of control diet (kcal/day) = Consumed food weight (g) × 3.04 (kcal/g)

Energy intake of HF (kcal/day) = Consumed food weight (g) × 5.12 (kcal/g)

(3) Calculation of body weight gain

The initial body weight and final body weight of each rat was represented by body weight on first day and last day before termination, respectively. The body weight gain per each rat was calculated as follows:

Body weight gain (g) = Final body weight (g) – Initial body weight (g)

3.2.4.5 Preparation and collection of blood and tissue samples

At the end of treatment term, the animals are anaesthetized and sacrificed by intraperitoneal injection of high dose (150 mg/kg) of sodium pentobarbital (nembutal) after a approximately 16-h fasting overnight period (Eu, Lim, Ton, & bin Abdul Kadir, 2010). The rats are cut open longitudinally from the top of the thorax to the pelvic region, exposing the internal organs. Under careful sterile conditions, the pancreas is promptly placed in a beaker containing cold (4 °C) phosphate-buffered saline or normal saline and gently cleaned of adhesive and contaminating tissue. Pancreas is quickly transferred to a sterile laminar flow hood. The dissections are carried out as rapidly as possible while maintaining care and precision. After the dissection, the pancreas is weighted. Pancreas is divided into five portions. The first portion is suspended in fresh RNAlater and the tissue is stored at -20 °C until RNA is isolated. The second portion is rapidly frozen in liquid nitrogen until the measurement of protein levels. The third portion is fixed in 10% formalin and the tissue is stored at room temperature for histological analysis. The fourth portion is fixed in 10% formalin and the tissue is stored at 4 °C for immunohistochemistry analysis. The last portion is stored at -20 °C for additional analysis in the future. Besides, other tissue or organ samples of interest, namely the heart, abdominal fat, epididymal fat pads, livers, and kidney are promptly harvested, rinsed with cold phosphate- buffered saline or normal saline, weighed and then stored in 10% formalin.

After opening the thorax, blood is drawn from the apex of cardiac ventricle (cardiac puncture). Blood samples are obtained into a sterile falcon tube and centrifuged at 460 × *g* for 20 min at 4 °C, and the serum supernatant are transposed to a new microcentrifuge tubes and stored at -20 °C prior to use for determination of lipid profile and insulin concentrations.

3.2.5 Blood biochemical measurement

The method and instruments for the determination of blood biochemical parameters are shown in Table 3.2.

Table 3.2

Summary of methods and instruments for determination of blood biochemical parameters

Blood biochemical parameters	Methods	Instruments
Glucose	Enzymatic oxidation of glucose	Glucosemeter
Lipid profile	Enzymatic-colorimetric method	Spectrophotometer
Liver function test	Kinetic UV method	Automate (Modular P8000)
Kidney function test	Enzymatic-colorimetric method	Automate (Architect)

3.2.5.1 Determination of serum lipid profile

(1) Determination of total-C

The levels of cholesterol in serum were determined using the enzymatic colorimetric method described by Richmond et al. in 1992. Cholesterol is detected enzymatically using cholesterol esterase, cholesterol oxidase and peroxidase. The stain intensity of quinieimine dye is straightly proportionate to cholesterol concentration and can be dictated photometrically. The reactions are as follows:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{FA}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinieimine dye} + 4H_2O$$

A 10 µL of serum samples and cholesterol calibrator (200 mg/dL or 5.17 mmol/L) was added to a 1000 µL of cholesterol reagent (R1 reagent), mixed and subsequently incubated for 10 min at room temperature. Cholesterol reagent consisted of pipes buffer (90 mmol/L), phenol (26 mmol/L), cholesterol esterase (300 U/L), cholesterol oxidase (200 U/L), peroxidase (1,250 U/L) and 4-

aminoantipyrine (0.4 mmol/L). Within 60 min read absorbance (A) of cholesterol calibrator and serum samples against reagent blank. The concentration of cholesterol was quantitated by spectrophotometer at the wavelength of 546 nm. The condensation of total-C is revealed as mg/dL. Cholesterol concentration was computed allowing to the following formula:

$$\text{Cholesterol concentration} = \frac{\Delta A \text{ sample} \times \text{Calibrator concentration}}{\Delta A \text{ calibrator}}$$

(2) Determination of HDL-C

The levels of HDL-C in serum were determined using the enzymatic colorimetric method. The chylomicrons, VLDL, and LDL-C are precipitated by phosphotungstic acid and magnesium chloride in precipitating reagent or HDL reagent. After centrifugation the supernatant includes the HDL-fraction, their cholesterol content is determined enzymatically. A 200 µL of serum samples was added to a 500 µL of working precipitating reagent (dilute 4 parts of precipitating reagent with 1 part of DW), mixed and subsequently incubated for 10 min at room temperature. The mixture was then centrifuged at 4,000 × *g* for 10 min at room temperature. After centrifugation, the mixture was separated the HDL-containing supernatant from the precipitated lipoprotein. A 100 µL of HDL supernatant was mixed with 1,000 µL of cholesterol reagent. The mixture was incubated for 10 min at room temperature. Then, the mixture was detected at 546 nm in the assay using the spectrophotometer. The concentration of HDL-C is expressed as mg/dL.Cholesterol concentration in HDL was calculated according to the following equation:

$$\text{Cholesterol in HDL concentration} = \frac{\Delta A \text{ sample} \times \text{Calibrator concentration} \times 3.5}{\Delta A \text{ calibrator}}$$

(3) Determination of TG

The levels of TG in serum were determined using the enzymatic colorimetric method described by Wahlefeld et al. in 1974. The TG is hydrolyzed by LPL to generate glycerol and FA. Glycerol is phosphorylated by glycerol kinase to produce the glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). The G-

3-P is converted by G-3-P peroxidase to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). The H_2O_2 is then reacted with 4-aminophenazone and 4-chlorophenol under the catalytic reaction of oxidase to give a red quinoneimine dye (Trinder endpoint reaction). This red dye stuff can be measured spectrophotometrically at 546 nm. The reactions are as follows:

$$TG + 3H_2O \xrightarrow{LPL} Glycerol + FA$$

$$Glycerol + ATP \xrightarrow{Glycerol\ kinase} G\text{-}3\text{-}P + ADP$$

$$G\text{-}3\text{-}P + O_2 \xrightarrow{G\text{-}3\text{-}P\ peroxidase} DAP + H_2O_2$$

$$H_2O_2 + 4\text{-aminophenazone} + 4\text{-chlorophenol} \xrightarrow{Peroxidase} 4\text{-}(\rho\text{-benzoguinone-monoimino})\text{-phenazone} + 2H_2O + HCl$$

A 10 µL of serum samples and TG calibrator (200 mg/dL or 2.28 mmol/L) was added to a 1,000 µL of TG reagent (R1 reagent), mixed and subsequently incubated for 10 min at room temperature. TG reagent consisted of pipes buffer (50 mmol/L), ρ-chlorophenole (2 mmol/L), lipoprotein lipase (150,000 U/L), glycerol kinase (800 U/L), G-3-P oxidase (4,000 U/L), peroxidase (440 U/L), 4-aminoantipyrine (0.7 mmol/L), ATP (0.30 mmol/L, magnesium chloride (40 mmol/L), Na-cholat (0.20 mmol/L) and potassium-hexacyanoferrate (ii) (1 µmol/L). Then read absorbance of serum samples and TG calibrator against reagent blank within 60 min after start. The concentration of TG was quantitated by spectrophotometer at the wavelength of 546 nm. The concentration of TG is expressed as mg/dL. TG concentration was calculated according to the following equation:

$$\text{TG concentration} = \frac{\Delta A\ \text{sample} \times \text{calibrator concentration}}{\Delta A\ \text{calibrator}}$$

(4) Determination of LDL-C

The concentration of LDL-C was expressed as mg/dL. The serum low-density lipoprotein-cholesterol (LDL-C) level was determined applying the Friedewald equation (Friedewald, Levy & Fredrickson, 1972), represented as follows:

LDL-C = (Total-C) – (HDL-C) – (Triglyceride/5)

3.2.5.2 Determination of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT)

At the end of the 4^{th} and 16^{th} weeks of the treatment, the levels of glucose in blood are tested using the blood glucose monitoring, Accu-Check. Rats are fasted overnight (16 h), weighed and clipped the tip tail for glycemic determinations. Blood samples are collected immediately prior to a glucose solution administration (at 0 min) for measurement of fasting glucose concentrations and at 30, 60, 90, 120 and 150 min after receiving of oral glucose loading (2 g/kg body weight) for the assay of oral glucose tolerance test. The glucose dehydrogenase enzyme in test strip, in the presence of the coenzyme, converts glucose in the blood sample to gluconolactone. A series of half-hourly blood glucose of each rats are analyzed and glucose concentrations are plotted against the time of blood withdrawals. Area under the curve (AUC) was calculated using trapezoidal rule (GraphPad Prism version 5.02, GraphPad). This method is used for determination of glucose concentrations and glucose tolerance as previously described with minor modification (Wolever, Jenkins, Jenkins, & Josse, 1991).

3.2.5.3 Determination of insulin concentrations

The animals are fasted for 16 h and blood samples are collected from cardiac puncture. Tubes were kept on ice until centrifugation at 1,040 × *g* for 5 min. Plasma was stored at -20 °C before being analyzed. Plasma insulin levels were analyzed by using the commercial ELISA kits. Mercodia Insulin ELISA is a intact stage two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed counter to separate antigenic determinants on the insulin particle. During incubation insulin in the sample responds with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies obligated to microtitration well. A simple washing proceeding removes unobligated

enzyme labelled antibody. The obligated conjugate is revealed by reaction with 3, 3', 5, 5'- tetramethylbenzidine (TMB). The reaction is halted by adding acid to produce a colorimetric endpoint that is interpret spectrophotometrically.

The concentrations of insulin are acquired by computerized data diminution of the absorbance for the calibrators, exclude for calibrator 0, versus the concentration using cubic spline regression by Prism GraphPad software.

3.2.5.4 Determination of liver and kidney function test

Serum aspartate aminotransferase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine were calculated by automate instrument. Serum liver function test and kidney function test were determined by kinetic UV method and enzymatic-colorimetric method, respectively.

3.2.6 Histological analysis of adipose and pancreas tissue

3.2.6.1 Preparation of tissue for histology

Tissue samples were fixed in buffered formalin (pH 7.4) for 12 h, labeled with the appropriate histology number and spotted in tissue cassettes. The cassettes were then spotted in an automated histology tissue processer and procedured overnight according to a set program (Table 3.3).

Table 3.3

Processing schedule for histological analysis

Carousel position	Reagent	Time (h)
1	10% formalin	12 h fixation
2	70% alcohol	1
3	80% alcohol	1
4	95% alcohol	2
5	95% alcohol	1
6	100% alcohol	2
7	100% alcohol	2
8	Xylene	1
9	Xylene	1
10	Xylene	1
11	Wax	2
12	Wax	3

The total running time for a complete cycle was 17 h.

3.2.6.2 Tissue embedding and sectioning

The tissue cassettes were removed from the processer and embedded in paraffin wax. Tissue sections, ranging between 3-5 µm in thickness, were then cut using a rotary microtome floated, onto warm (40-45 °C) water to remove wrinkles and placed onto slides. Stored slides in racks in an upright position, then dried in an oven, temperatures should not exceed 65 °C for 30 min (Rolls, 2008).

3.2.6.3 Hematoxylin and eosin staining

Samples of adipose and pancreas tissue obtained from rats are stained with hematoxylin and eosin (Luna, 1968; Ann Preece, 1972; Horobin, 1977). The process of staining is shown in figure 3.1. The samples are then examined for morphological observation under a light microscope (Olympus CX31) equipped with a digital camera. Area of the adipocyte is measured by Axiovision AC. Mean adipocyte area is calculated from a 100 cells observed/μm^2.

Table 3.4

H & E staining protocol

Step	Procedure	Time (min)
1	Xylene	3
2	Xylene	2
3	100% alcohol	3
4	100% alcohol	2
5	95% alcohol	3
6	95% alcohol	2
7	80% alcohol	3
8	Hematoxylin	3
9	95% alcohol	3
10	Eosin	1
11	95% alcohol	3
12	95% alcohol	2
13	100% alcohol	2
14	100% alcohol	2
15	Xylene	2

The total running time for a complete cycle was 36 min.

3.2.7 Immunohistochemistry (IHC) staining of pancreas

3.2.7.1 Immunolabelling for SA-β-galactosidase in the rat pancreas

Senescence-associated β-galactosidase (SA-β-gal) is a supposed hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides only in senescent cells. SA-β-gal was identified by immunostaining using anti-β-galactosidase (polyclonal mouse anti-β-galactosidase, Abnova, Jhouzih St., Taipei, Taiwan). Formalin fixed paraffin embedded tissues were cut at 3 µm and baked at 60 °C for 30 min prior to IHC. Antigens were retrieved using hyaluronidase (APPENDIX F). Peroxidase quenching was performed by using 3% H_2O_2 in methanol for 30 min at 37 °C. The non-specific binding site was blocked with 5% BSA in 1×PBST (0.1% Tween in 1×PBS) (APPENDIX F). The section slides were incubated

with 1:100 β-galactosidase primary antibodies overnight at 4 °C. After rinsing, the sections were incubated at a dilution of 1:1000 horseradish peroxide donkey anti-rabbit IgG for 30 min at 37 °C in the moisture chamber. Immunostaining was visualized using liquid diaminobenzidine tetrachloride (DAB) chromogen. An insoluble brown precipitate of the DAB reaction developed at the β-galactosidase antibody/antigen binding site. It was counterstained with Mayer's Hematoxylin. After being air dried, the sections were mounted with Entellan® and overlaid with a cover slip.

3.2.7.2 Image analysis of immunolabeling for SA β-galactosidase

The stained sections of pancreas were captured using the ×10, ×40 and ×100 objective attached to an Olympus BX60 light microscope equipped with a digital camera (Nikon, Japan) and interfaced with NIH Image 1.63 software. The mean density of SA-β-gal in islet areas was analyzed using the NIH program.

3.2.8 Determination of pancreatic mRNA expression

The differential displays of pancreatic mRNA for IRS-2 and NF-κB are measured by quantitative real-time polymerase chain reaction (real-time PCR) analysis.

3.2.8.1 Total RNA isolation and purification

In brief, total RNA is extracted from excised rat pancreas using guanidinium thiocyanate-phenol-chloroform method according to the manufacturer's recommendations. Pancreas is homogenized by sterile mortar on liquid nitrogen. TRIzol reagent (TRIzol reagent contains: phenol, guanidinium thiocyanate and other components) and chloroform are added to homogenate and subsequently centrifuges the mixture at 12,000 × *g* for 15 min at 4 °C. The RNA contains in the aqueous phase and places to 2-propanol (isopropanol). The mixture is centrifuged at 12,000 × *g* for 10 min at 4 °C and removed the supernatant from the tube. The RNA pellet is washed with 75% ethanol and then centrifuged at 75,000 × *g* for 10 min at 4 °C. The RNA pellet is dried for 10-15 min at room temperature. Dried RNA is dissolved with diethylpyrocarbonate (DEPC)-treated water or RNase-free water. Total RNA concentrations are determined using the NanoDrop 2000 spectrophotometer by deciding the absorbance values at 260 nm and 280 nm (A260/A280 ratio) where a ratio of 1.9-2.1 is examined pure.

3.2.8.2 Complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA is reverse transcribed to synthesize single stranded cDNA using the High Capacity cDNA Reverse Transcription Kits. The reaction components for cDNA synthesis are shown in Table 3.5. The MyCycler™ thermal cycler conditions are as follows: initial set up cycle at 25 °C for 10 min and 37 °C for 120 min, resulted by cycles at 85 °C for 5 min, and 4 °C for infinity (Table 3.5). Each cDNA solution was stored at -80 °C.

Table 3.5

Reaction components for cDNA synthesis

Reaction components	Volume/reaction (µL)
	Kit with RNase inhibitor
2× RT master mix:	
1) DEPC-treated water	3.2
2) 10× RT buffer	2
3) 10× RT random pimers	2
4) 25× dNTP Mix (100mM)	0.8
5) RNase Inhibitor	1
6) MultiScribe Reverse Transcriptase	1
Total per reaction	10
cDNA RT reaction:	
1) 2× RT master mix	10
2) RNA sample	10
Total per reaction	20

Table 3.6

Incubating protocol for cDNA synthesis

	Step 1 (primer extension)	Step 2 (cDNA synthesis)	Step 3 (reaction termination)	Step 4 (hold)
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

3.2.8.3 Quantitative real-time PCR

Total real-time PCR reaction system is perfumed in StepOne™ Real-Time PCR System or real-time PCR instrument. PCR products are quantitatively constituted from cDNA samples using the TaqMan® Gene Expression Master Mix (TaqMan Gene Expression Master Mix contains: AmpliTaq Gold® DNA Polymerase, Ultra Pure, Uracil-DNA glycosylase, deoxyribonucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP), ROX Passive Reference and Optimized buffer components) and TaqMan® Gene Expression Assay. Relative mRNA levels are measured using the method before via TaqMan analysis that employed gene-specific primers and probes (FAM™ dye-labeled MGB probe). Each reaction contained 4 μL of cDNA solution (40 ng), 10 μL of TaqMan Gene Expression Master Mix, 1.5 μL of TaqMan® Gene Expression Assay and 4.5 of μL DEPC-treated water in a final volume of 20 μL (Table 3.7). The oligonucleotide sequences of primers and probes for TaqMan analysis of IRS-2, NF-κB and β-actin are all obtained from TaqMan® Gene Expression Assays, β-actin is used as internal control. The relative expression levels of each gene are decided by the comparative 2 AACt method. Cycling conditions are as follows: initial set up cycle at 50 °C for 2 min and 95 °C for 10 min, imitated by 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. To allow for comparisons between samples and groups, quantities of all targets in test samples are normalized to the constitutive housekeeping gene β-actin. The cycling program used is presented in Table 3.7.

Table 3.7

Reaction components for real-time PCR

Reaction components	Volume/reaction
1) TaqMan Gene Expression Master Mix	10 μL
2) TaqMan Gene Expression Assays (target genes or reference gene)	1.5 μL
3) DEPC-treated water	4.5 μL
4) cDNA	4 μL
Reaction volume/final volume	20 μL
Total amount of cDNA	40 ng

Table 3.8

Cycling protocol for real-time PCR

Step	UDG incubation	AmpliTaq Gold, UP enzyme activation	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/extend
Temperature (°C)	50	95	95	60
Time	2 min	10 min	15 s	1 min

Relative mRNA levels were calculated using the method before via TaqMan analysis that used gene-specific primers and probes [FAM dye-labeled minor groove binder (MGB) probe or TaqMan MGB probe]. TaqMan MGB probes composed of reporter dye (FAM dye) and nonfluorescent quencher (NFQ)-linked MGB at 5´and 3´ end, respectively. The quencher dye has a suppressible activity to fluorescence in the reporter. During PCR, the reporter dye was separated from the quencher dye by 5´ exonuclease action of the DNA polymerase. The releasing of the reporter dye results in elevated fluorescence. This elevation was detected for determination of real-time PCR products (APPENDIX G). The real-time PCR results were quantified base on the enumerate of cycles required for amplification-produced fluorescence to touch a definite threshold of detection (the threshold cycle or C_T value). The relative mRNA expression regulars of each gene were analyzed by the

comparative $2^{-\Delta\Delta CT}$ method. The expression levels of β-actin were used for normalization. The following steps were used to calculate relative quantitation (RQ) value:

Step 1: calculate the ΔC_T

$$\Delta C_{T(\text{test/calibrator sample})} = C_{T(\text{target gene, test/calibrator sample})} - C_{T(\text{reference gene, test/calibrator sample})}$$

Step 2: calculate the $\Delta\Delta C_T$

$$\Delta\Delta C_{T(\text{test/calibrator sample})} = \Delta C_{T(\text{test/calibrator sample})} - \Delta C_{T(\text{calibrator sample})}$$

Step 3: calculate the RQ value

$$RQ = 2^{-\Delta\Delta CT}$$

3.3 Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.). All other data were analyzed using statistics package for the social sciences (SPSS) for windows version 13.0. The comparison between two groups was analyzed by unpaired two-tailed Student's t-test. Data is analyzed between group using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. Statistically significant difference is considered as a p-value less than 0.05.

CHAPTER 4
RESULTS

4.1 Total phenolic contents and DPPH radical-scavenging activity of BWE and WWE

The yield of BWE and WWE crude extract were 6.67% and 3.33% w/w, respectively. Proximate analysis of 100 g BWE; (1) Energy 244 kcal, (2) Energy from fat 2 kcal, (3) Carbohydrate 46.32 g, (4) Protein 14.20 g, (5) Fat 0.20 g, (6) Ash 25.83 g, (7) Humidity 13.45 g (APPENDIX H). Proximate analysis of 100 g WWE; (1) Energy 264 kcal, (2) Energy from fat 3 kcal, (3) Carbohydrate 51.68 g, (4) Protein 13.62 g, (5) Fat 0.32 g, (6) Ash 24.44 g, (7) Humidity 9.94 g (APPENDIX I).The content of total phenolic compounds were 34.06 ± 0.61 mg GAE/g of BWE and 44.44 ± 1.12 mg GAE/g of WWE, respectively (Table 4.1). The EC_{50} of the BWE and WWE in scavenging DPPH radical were 57.98 ± 10.52 µg/mL and 31.73 ± 0.42 µg/mL, respectively whereas EC_{50} for BHT was 11.60 ± 1.99 µg/mL (Table 4.1).

Table 4.1

Total phenolic contents and DPPH radical-scavenging activity of BWE and WWE

	Concentrations
A. Total phenolic contents	
BWE	34.06 ± 0.61 mg GAE/g of BWE
WWE	44.44 ± 1.12 mg GAE/g of WWE
B. DPPH radical-scavenging activity	
BWE	57.98 ± 10.52 µg/mL
WWE	31.73 ± 0.42 µg/mL
BHT	11.60 ± 1.99 µg/mL

Values are expressed as mean ± S.E.M. (n = 3)

4.2 Effects of BWE on diet, energy intake and body weights

4.2.1 Short-term experiment

Data of daily dietary intakes and body weight in all short-term groups are shown in Table 4.2. The control animals consumed significantly more daily food intakes than the HF-fed animals. On the other hand, the energy intakes were significantly higher in all HF-fed groups than that of the standard chow-fed group. Daily food and energy intakes had no changes between untreated HF and treated HF groups. At the beginning of the experimental period, there were no differences between each experimental groups for the mean body weight of rats. After 4 weeks, body weight and body weight gain of rats were significantly increased in all HF groups compared with the control group, but no significance difference between untreated HF and treated HF groups

Table 4.2

Effects of BWE on daily dietary/energy intakes and body weights in short-term experiment

Parameters	Short-term groups					
	Control	HF	HFB1	HFB10	HFW	HFM
A. Dietary intake						
Food intake (g/day)	24.57 ± 0.30	22.27 ± 0.28*	21.45 ± 0.24*	20.85 ± 0.57*	21.44 ± 0.30*	21.29 ± 0.80*
Energy intake (kcal/day)	74.75 ± 0.98	113.88 ± 1.48*	109.88 ± 1.26*	108.50 ± 2.91*	109.75 ± 1.45*	109.00 ± 4.13*
B. Body weight						
Initial BW (g)	241.66 ± 2.76	241.25 ± 3.46	241.63 ± 3.07	243.13 ± 3.42	240.88 ± 3.63	242.25 ± 3.86
Final BW (g)	367.45 ± 4.60	414.38 ± 12.12*	414.13 ± 8.82*	399.63 ± 6.59*	411.75 ± 6.25*	400.25 ± 11.37*
BW gain (g)	125.79 ± 3.55	173.13 ± 9.01*	172.50 ± 7.54*	156.50 ± 7.94*	170.88 ± 5.89*	158.00 ± 8.33*
BW gain (%)	50.33 ± 2.97	71.54 ± 2.85*	71.40 ± 3.02*	64.64 ± 3.77*	71.11 ± 2.82*	65.07 ± 2.74*

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.2.2 Intermediate-term experiment

Data of daily dietary intakes and body weight in all intermediate-term groups are shown in Table 4.3. The control group consumed significantly more daily food intake than the HF group. However, the energy intakes were significantly higher in both HF and HFB1 groups as compared with the control group. The initial body weight in rats before treatment was not significantly different in all experimental groups. After sixteen weeks, final body weight and body weight gain were significantly higher in the HF and HFB1 groups when compared to the control group.

Table 4.3

Effects of BWE on daily dietary/energy intakes and body weights in intermediate-term experiment

Parameters	Intermediate-term groups		
	Control	HF	HFB1
A. Dietary intake			
Food intake (g/day)	24.29 ± 0.23	19.11 ± 0.13*	19.02 ± 0.30*
Energy intake (kcal/day)	73.75 ± 0.75	97.63 ± 0.73*	97.25 ± 1.53*
B. Body weight			
Initial BW (g)	244.00 ± 3.37	240.38 ± 2.25	245.63 ± 3.65
Final BW (g)	530.38 ± 2.75	599.25 ± 6.79*	590.38 ± 7.38*
BW gain (g)	289.75 ± 5.74	358.38 ± 6.19*	346.38 ± 11.41*
BW gain (%)	119.42 ± 4.16	149.67 ± 3.39*	141.77 ± 6.98*

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.3 Effects of BWE on obesity

4.3.1 Short-term experiment

Relative organ weights of rats in the short-term groups are shown in Table 4.4. After four weeks of treatment period, the relative weights of abdominal fat and epididymal fat tissues were significantly increased in the HF group as compared with the control group. The abdominal fat of HFB1, HFB10 and HFM were significantly decreased as compared with the HF group. The epididymal fat of only HFB10 group was significantly decreased as compared with the HF group.

Table 4.4

Effects of BWE on relative organ weights in short-term experiment

Parameters	Short-term groups					
	Control	HF	HFB1	HFB10	HFW	HFM
Abdominal fat (g/100 g BW)	0.95 ± 0.08	2.06 ± 0.11*	1.72 ± 0.16*,†	1.49 ± 0.15*,†	2.03 ± 0.11*	1.26 ± 0.07†
Epididymal fat (g/100 g BW)	1.23 ± 0.07	1.92 ± 0.04*	1.82 ± 0.11*	1.59 ± 0.11*,†	1.89 ± 0.15*	1.99 ± 0.12*

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.3.2 Intermediate-term experiment

Compared to the control group, the relative weight of abdominal fat and epididymal fat tissues in HF group were significantly increased. The relative weights of abdominal fat and epididymal fat tissues in HFB1 group were not significantly differences compared with the HF group (Table 4.5).

Table 4.5

Effects of BWE on relative organ weights in intermediate-term experiment

Parameters	Intermediate-term groups		
	Control	HF	HFB1
Abdominal fat (g/100 g BW)	2.14 ± 0.30	3.31 ± 0.15*	2.81 ± 0.17
Epididymal fat (g/100 g BW)	2.04 ± 0.17	2.79 ± 0.12*	2.45 ± 0.09

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.4 Effects of BWE on histology of the epididymal fat pads

4.4.1 Short-term experiment

According to H & E staining, the epididymal adipocyte size in the HF-fed group was markedly larger than that of the control group. Conversely, the adipocyte size was significantly decreased in the HFB1, HFB10, HFW and HFM groups when compared to the HF group (Figure 4.1 and 4.3).

4.4.2 Intermediate-term experiment

H & E-stained epididymal fat pads, the adipocyte size was greater in rats fed with HF alone than in standard chow-fed rats (Figure 4.2 and 4.3). The adipocyte size of HFB1 was significantly decreased as compared with the HF group.

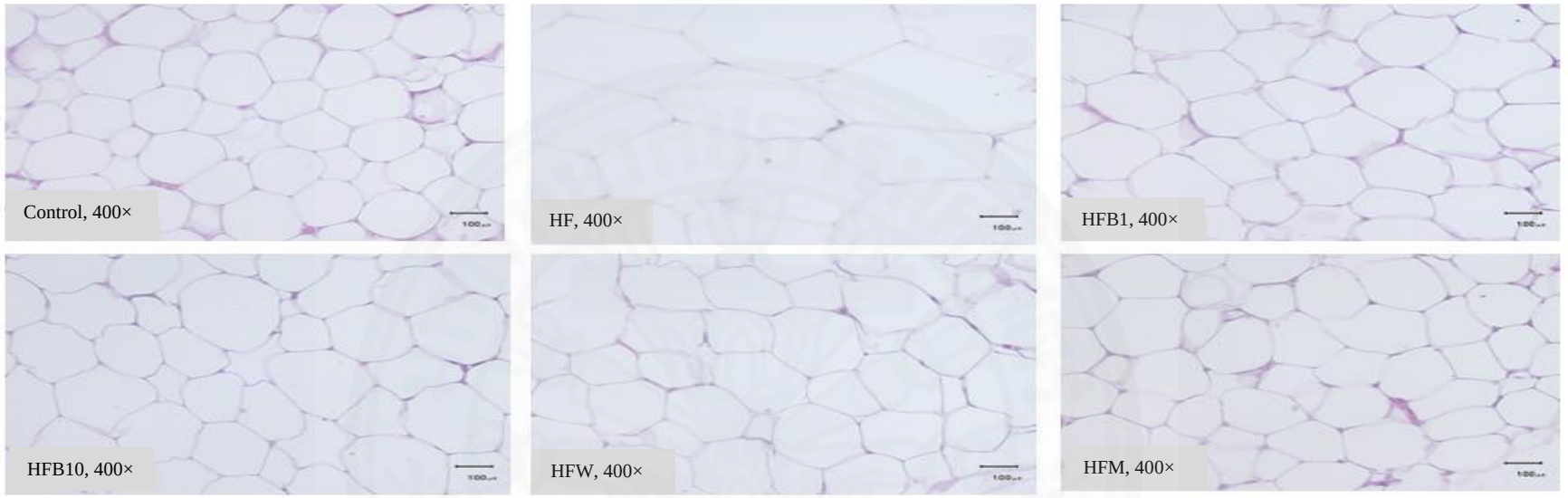


Figure 4.1 Histology of epididymal fat pads in the short-term groups (H & E staining, magnification 400×, scale bar = 100 μm) (n = 3).

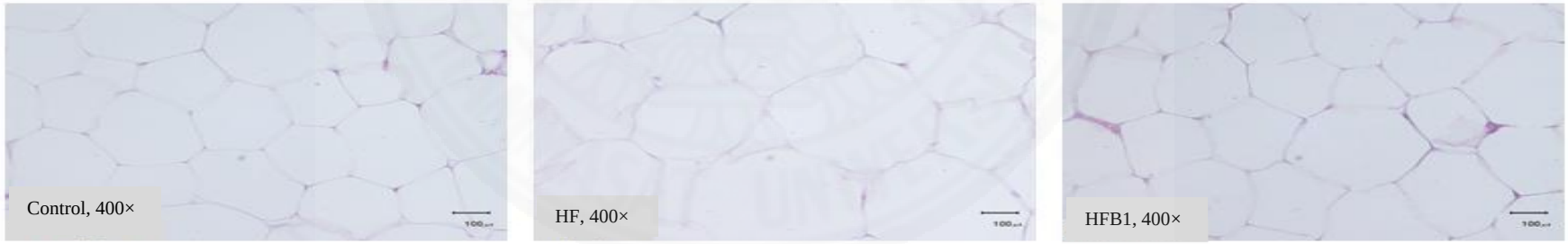
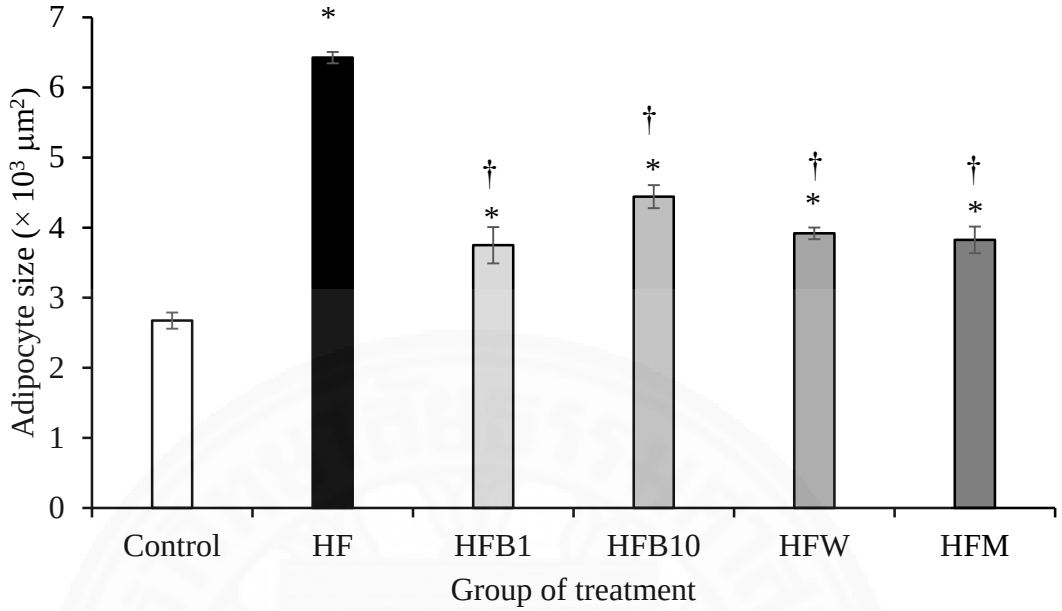


Figure 4.2 Histology of epididymal fat pads in the intermediate-term groups (H & E staining, magnification 400×, scale bar = 100 μm) (n = 3).

(a)



(b)

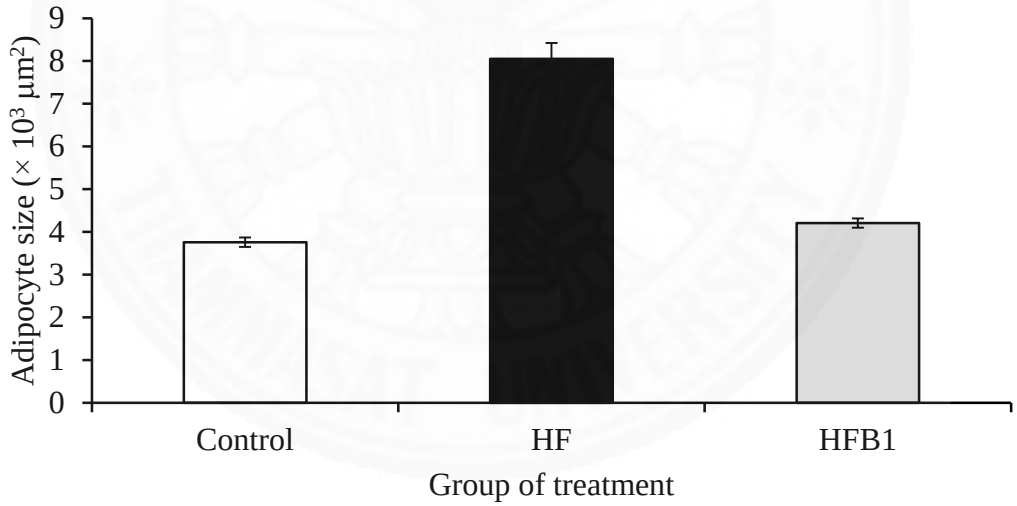


Figure 4.3 Adipocyte size in the short-term groups (a) and intermediate-term group (b). Values are expressed as mean ± SEM (n = 3).

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.5 Effects of BWE on serum lipid profile

4.5.1 Short-term experiment

Serum lipid profile is shown in Table 4.6. Evaluation of total cholesterol and TG were found in high-fat fed rats compared with normal diet control. Treatment with low dose BWE (HFB1 group) significantly reduced total cholesterol and LDL-C level compared with high-fat fed rats. HDL-C was significantly increased in only HFB1-treated group compared to HF-fed rats. The serum TG levels of HFB10 and HFW groups were significantly lower than HF group. The serum total cholesterol and TG levels of metformin-treated rats were significantly lower than those of the HF-fed rats.

Table 4.6

Effects of BWE on serum lipid profile in short-term experiment

Parameters	Short-term groups					
	Control	HF	HFB1	HFB10	HFW	HFM
Total cholesterol (mg/dL)	76.19 ± 2.02	95.73 ± 2.79*	79.41 ± 2.95†	94.13 ± 4.20*	96.55 ± 3.72*	74.44 ± 1.43†
LDL-cholesterol (mg/dL)	32.21 ± 5.33	40.58 ± 4.68	24.33 ± 1.72†	41.84 ± 2.89	43.84 ± 3.04*	30.29 ± 3.82
HDL-cholesterol (mg/dL)	38.53 ± 3.01	37.84 ± 3.53	46.86 ± 1.97*,†	44.05 ± 2.18	43.01 ± 0.99	44.54 ± 2.30
TG (mg/dL)	28.70 ± 1.56	40.73 ± 1.77*	36.66 ± 2.49*	33.03 ± 2.51†	33.40 ± 1.48†	32.52 ± 1.02†

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.5.2 Intermediate-term experiment

The significantly increased levels of total cholesterol, LDL-C and significantly decreased levels of HDL-C and TG were observed in the HF group compared to control group (Table 4.7). However, there were no significant differences in total cholesterol, LDL-C, HDL-C and TG between HF and HFB1 groups.

Table 4.7

Effects of BWE on serum lipid profile in intermediate-term experiment

Parameters	Intermediate-term groups		
	Control	HF	HFB1
Total cholesterol (mg/dL)	94.86 ± 2.19	107.23 ± 3.66*	100.21 ± 4.95
LDL-cholesterol (mg/dL)	33.20 ± 1.88	50.11 ± 3.06*	54.50 ± 5.62*
HDL-cholesterol (mg/dL)	46.39 ± 0.92	40.85 ± 0.88*	39.31 ± 1.28*
TG (mg/dL)	71.39 ± 3.91	56.43 ± 4.07*	56.95 ± 2.75*

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.6 Effects of BWE on glucose homeostatic parameters

4.6.1 Short-term experiment

The parameters of glucose homeostasis are shown in Table 4.8. In the short-term study, FBG and AUC-G in rat fed high-fat were higher than that of control. There were no significant difference in the fasting serum insulin between the six groups. There were no significant difference between FBG, AUC-G and fasting serum insulin between HFB1, HFB10, HFW and HFM compared with control group.

Table 4.8

Effects of BWE on glucose homeostatic parameters in short-term experiment

Parameters	Short-term groups					
	Control	HF	HFB1	HFB10	HFW	HFM
Fasting blood glucose (mg/dL)	108.38 ± 2.58	122.63 ± 1.95*	123.88 ± 1.65*	116.63 ± 3.29*	121.13 ± 1.93*	117.75 ± 1.83*
AUC-G (mg/dL.min)	3616 ± 322	4483 ± 238*	4126 ± 347	3961 ± 352	4433 ± 344	4202 ± 259
Fasting serum insulin (mU/L)	35.98 ± 3.65	35.94 ± 8.57	35.22 ± 3.29	31.00 ± 4.19	45.06 ± 5.05	35.53 ± 6.28

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.6.2 Intermediate-term experiment

As shown in Table 4.9, there was no significant difference in FBG among the groups. However, AUC-G was significantly higher in HFB1 group when compared with control group.

Table 4.9

Effects of BWE on glucose homeostatic parameters in intermediate-term experiment

Parameters	Intermediate-term groups		
	Control	HF	HFB1
Fasting blood glucose (mg/dL)	109.38 ± 3.62	111.25 ± 2.10	106.13 ± 1.77
AUC-G (mg/dL.min)	1581 ± 204	2609 ± 418	3528 ± 602*
Fasting serum insulin (mU/L)	56.47 ± 5.27	61.38 ± 6.80	54.56 ± 4.43

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.7 Effects of BWE on OGTT

4.7.1 Short-term experiment

There were no differences in blood glucose concentrations at 30 min of OGTT in all groups. At 60 min, blood glucose concentrations were found to be significantly increased in HF, HFB1, HFB10, and HFM groups when compared to the control group (Figure 4.4). At 90 min, blood glucose concentrations were found to be significantly increased in the HFB1 group when compared to the control group (Figure 4.4). At 120 min, blood glucose concentrations of the HF group were higher than the other groups while the blood glucose concentrations of the HFB10- and HFM-fed rats showed a significant decrease when compared to the HF rats (Figure 4.4). At 150 min, blood glucose concentrations were significantly increased in the HFB10 and HFM groups compared to the control group. Thus, it can be concluded that the blood glucose concentrations showed a significant decrease in the HFB10 and HFM-fed rats when compared to the HF rats (Figure 4.4).

4.7.2 Intermediate-term experiment

There were no differences in blood glucose concentrations at 30, 90, 120 and 150 min of OGTT in three groups. At 60 min, blood glucose concentrations were found to be significantly increased in HF and HFB1 groups compared to the control group. Blood glucose concentrations were found to be not significantly difference between HF and HFB1 groups (Figure 4.5).

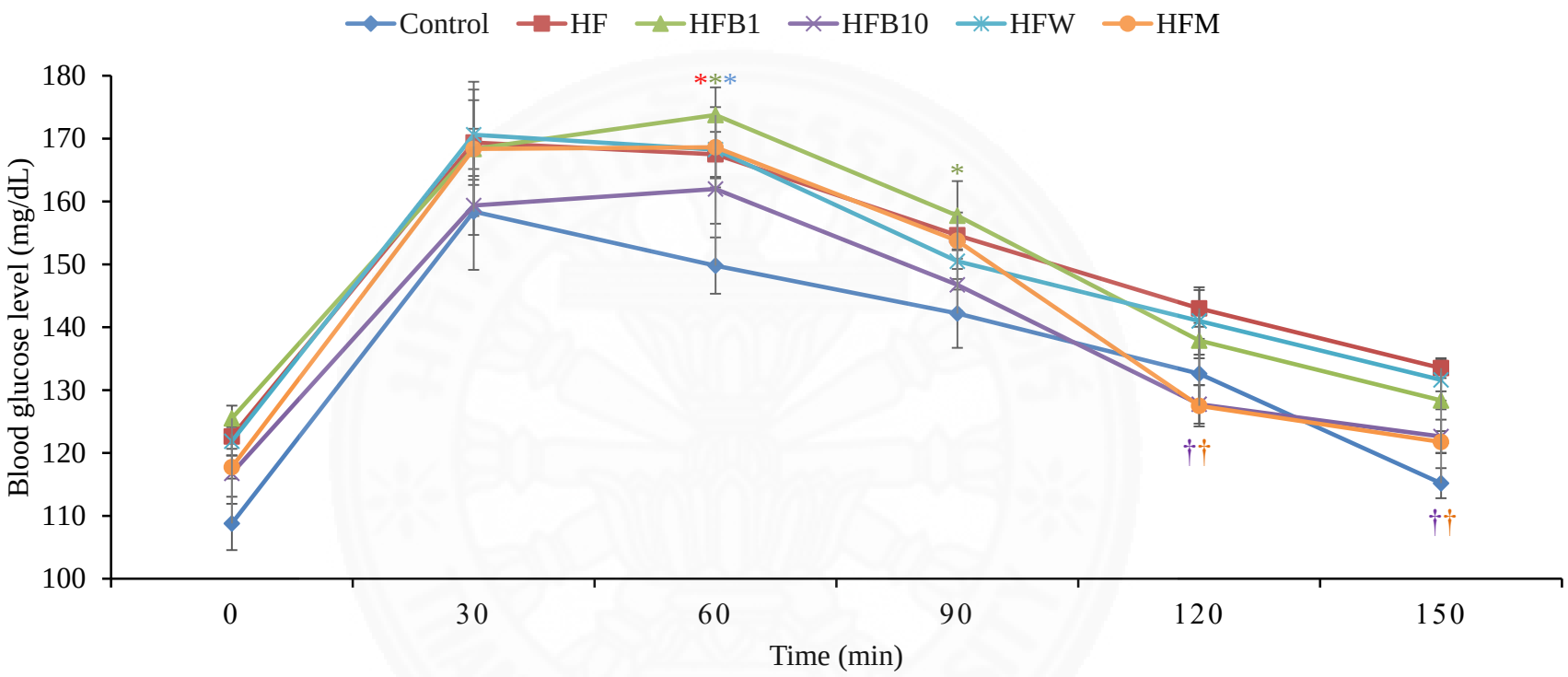


Figure 4.4. Oral glucose tolerance test of the short-term groups. Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

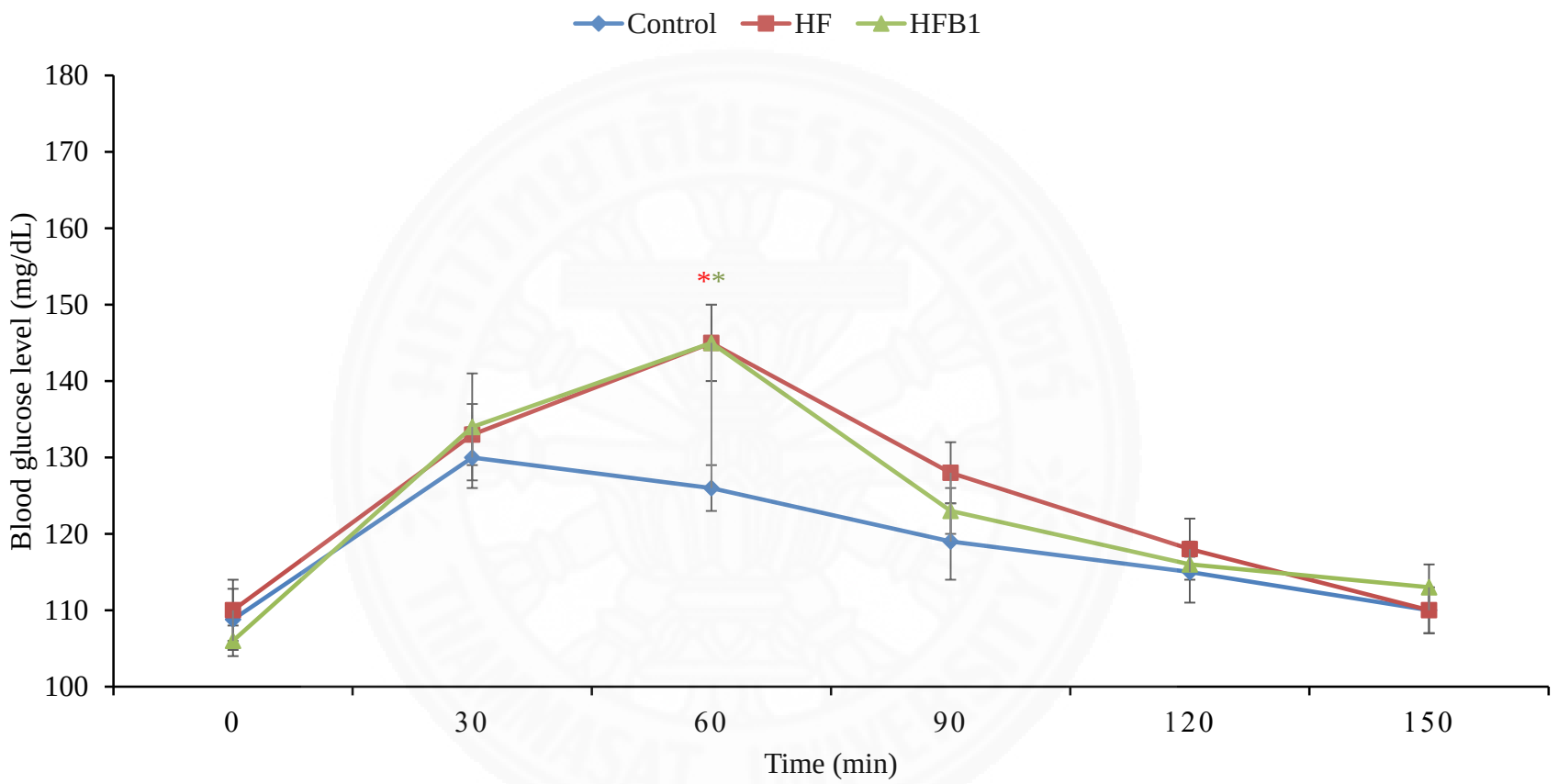


Figure 4.5. Oral glucose tolerance test of the intermediate-term groups. Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.8 Effects of BWE on liver function test and kidney function test

4.8.1 Short-term experiment

Serum AST, ALT, BUN and creatinine levels in rats did no differences among the experimental groups (Table 4.10).

Table 4.10

Effects of BWE on liver and kidney function test in short-term experiment

Parameters	Short-term groups					
	Control	HF	HFB1	HFB10	HFW	HFM
A. Liver function test						
AST (U/L)	89.00 ± 3.61	89.00 ± 6.71	89.00 ± 3.65	97.00 ± 5.45	88.00 ± 4.49	89.00 ± 6.45
ALT (U/L)	41.00 ± 1.97	37.00 ± 1.69	39.00 ± 1.04	38.00 ± 0.98	39.00 ± 1.50	37.00 ± 1.60
B. Kidney function test						
BUN (mg/dL)	14.00 ± 0.75	13.00 ± 0.53	13.00 ± 0.48	14.00 ± 0.53	13.00 ± 0.89	13.00 ± 0.65
Creatinine (mg/dL)	0.40 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.02

Values are expressed as mean ± SEM (n = 8)

4.8.2 Intermediate-term experiment

As shown in Table 4.11, there were no differences in AST, ALT, BUN and creatinine among the groups.

Table 4.11

Effects of BWE on liver and kidney function test in intermediate-term experiment

Parameters	Intermediate-term groups		
	Control	HF	HFB1
A. Liver function test			
AST (U/L)	102.00 ± 7.79	94.00 ± 8.74	102.00 ± 8.26
ALT (U/L)	45.00 ± 1.96	45.00 ± 1.10	44.00 ± 1.60
B. Kidney function test			
BUN (mg/dL)	17.00 ± 0.38	16.00 ± 0.53	16.00 ± 0.56
Creatinine (mg/dL)	0.40 ± 0.02	0.40 ± 0.02	0.40 ± 0.02

Values are expressed as mean ± SEM (n = 8)

4.9 Effects of BWE on pancreas

4.9.1 Effects of BWE on pancreatic mass

4.9.1.1 Short-term experiment

The relative weights of pancreas of HF, HFB1 and HFW groups were significantly decreased when compared with control group. An oral administration of metformin caused a significant increase in this parameter when compared to HF group. The relative weights of pancreas of HFM group were significantly increased when compared with HF group. (Table 4.12).

Table 4.12

Effects of BWE on relative weight of pancreas in short-term experiment

Parameters	Short-term groups					
	Control	HF	HFB1	HFB10	HFW	HFM
Pancreas (g/100 g BW)	0.56 ± 0.01	0.45 ± 0.02*	0.44 ± 0.01*	0.48 ± 0.03	0.43 ± 0.02*	0.53 ± 0.05†

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.9.1.2 Intermediate-term experiment

The relative weights of pancreas of HF and HFB1 groups were significantly decreased when compared with control group. There were found to be no differences between HF and HFB1 groups (Table 4.13).

Table 4.13

Effects of BWE on relative weight of pancreas in intermediate-term experiment

Parameters	Intermediate-term groups		
	Control	HF	HFB1
Pancreas (g/100 g BW)	0.40 ± 0.03	0.31 ± 0.02*	0.33 ± 0.02*

Values are expressed as mean ± SEM (n = 8)

* $p < 0.05$ vs. control

4.9.2 Effects of BWE on histology of the pancreatic acinar cells

4.9.2.1 Short-term experiment

The intracytoplasmic vacuoles (Figure 4.6, black arrows) were observed numerous in the pancreatic acinar cells of the HF group; however, intracytoplasmic vacuoles were found lesser than in HFB1, HFB10 and HFW groups (black arrows). The intracytoplasmic vacuoles were not found in the pancreatic acinar cells of the rats fed with the metformin and control group.

4.9.2.2 Intermediate-term experiment

Examination of H & E-stained slides from all HF-fed rats showed an increase of intracytoplasmic vacuoles in pancreatic acinar cells compared to normal control diet-fed rats. The intracytoplasmic vacuoles in pancreatic acinar cells of HFB1 group were found lesser than HF group (Figure 4.7, black arrows).

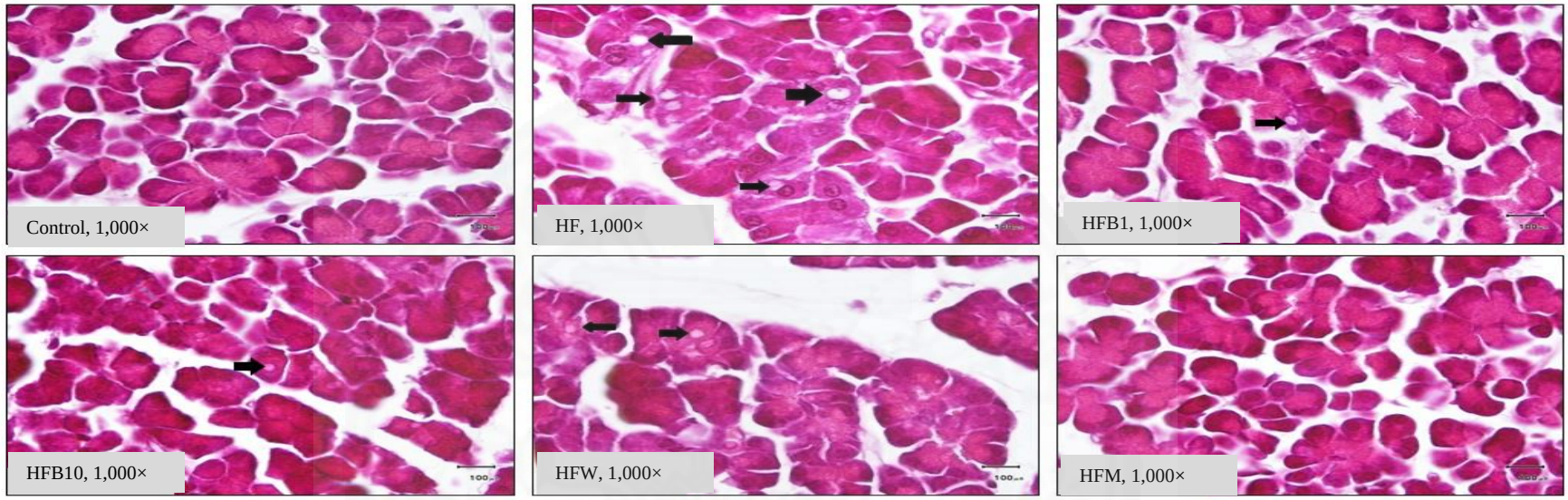


Figure 4.6 Histology of pancreatic acinar cells in the short-term groups (H & E staining, magnification 1,000×, scale bar = 100 µm), arrow represents intracytoplasmic vacuole in acinar cells (n = 3).

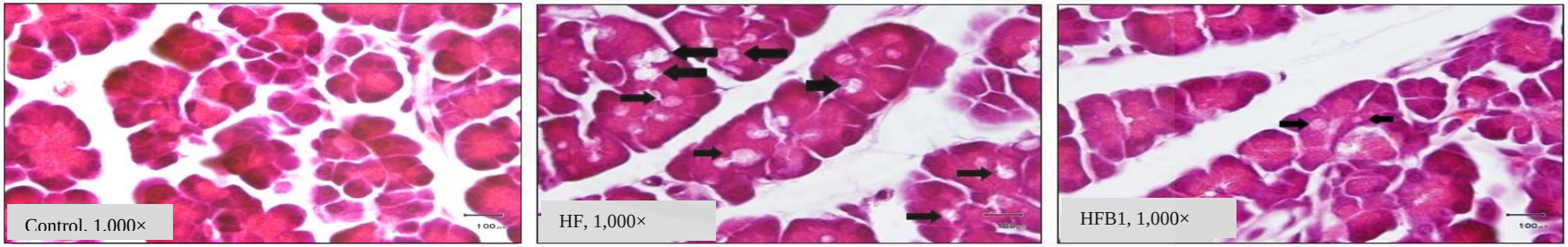


Figure 4.7 Histology of pancreatic acinar cells in the intermediate-term groups (H & E staining, magnification 1,000×, scale bar = 100 µm), arrow represents intracytoplasmic vacuole in acinar cells (n = 3).

4.9.3 Effects of BWE on histology of the islets of Langerhans

4.9.3.1 Short-term experiment

Islets of Langerhans from control group exhibited normal feature with no pathological lesions (Figure 4.8). The HF rats possessed a decreased number of islets and showed an enlarged islet area (Figure 4.8). The number of islets in the HFM group was significantly higher ($p < 0.05$) than in the HF group. Likewise, there was a trend toward an increase in the number of islets in the HFB1, HFB10, and HFW-treated rats compared with the HF rat, but the differences were not significant. The area of islets of the HF group was significantly higher ($p < 0.05$) than in the control group (Figure 4.9).

4.9.3.2 Intermediate-term experiment

There was no significant difference of the number of islets and islet size in all groups of treatments (Figure 4.10 and 4.11).

(a) Magnification ×200 (b) Magnification ×400

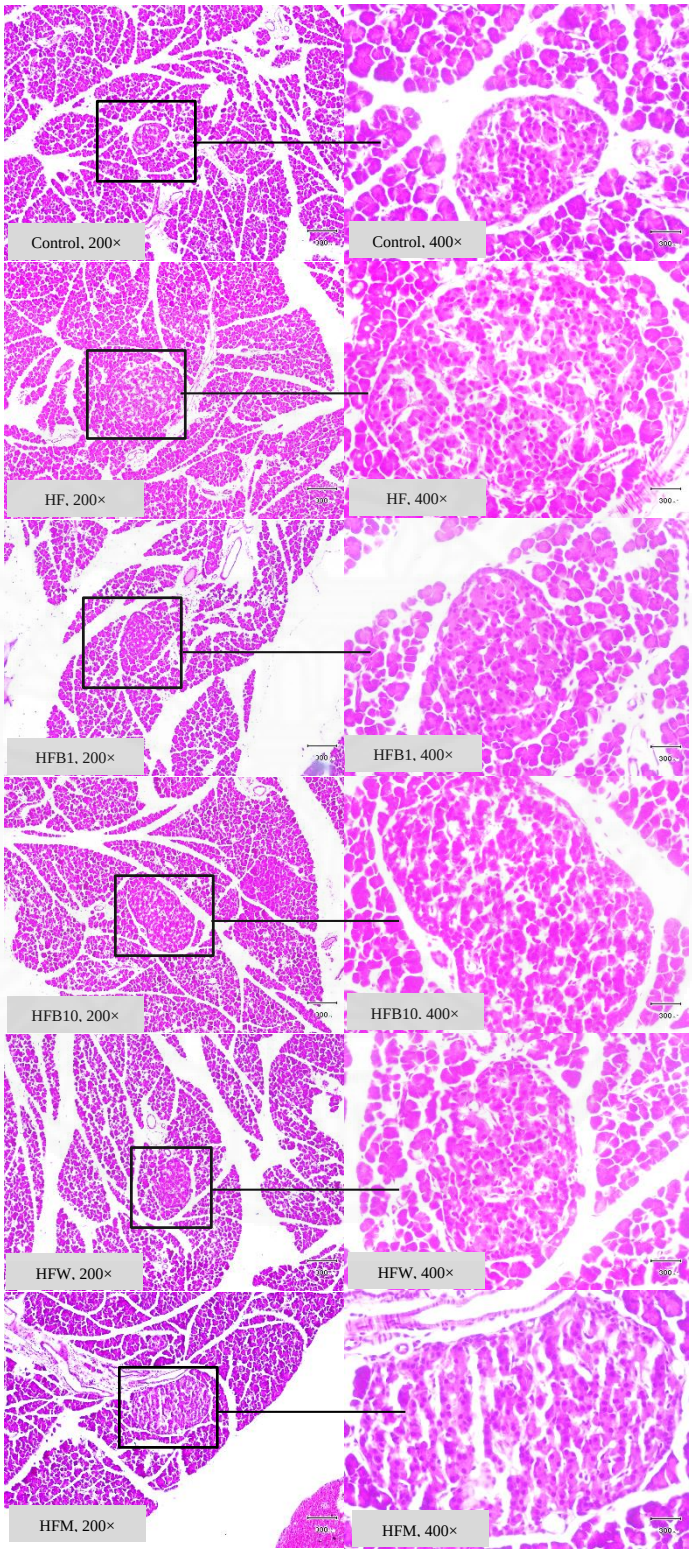


Figure 4.8 Histology of pancreatic islets of Langerhans (H & E staining, magnification 200× (a), 400× (b), scale bar = 100 µm) of short-term groups (n = 3).

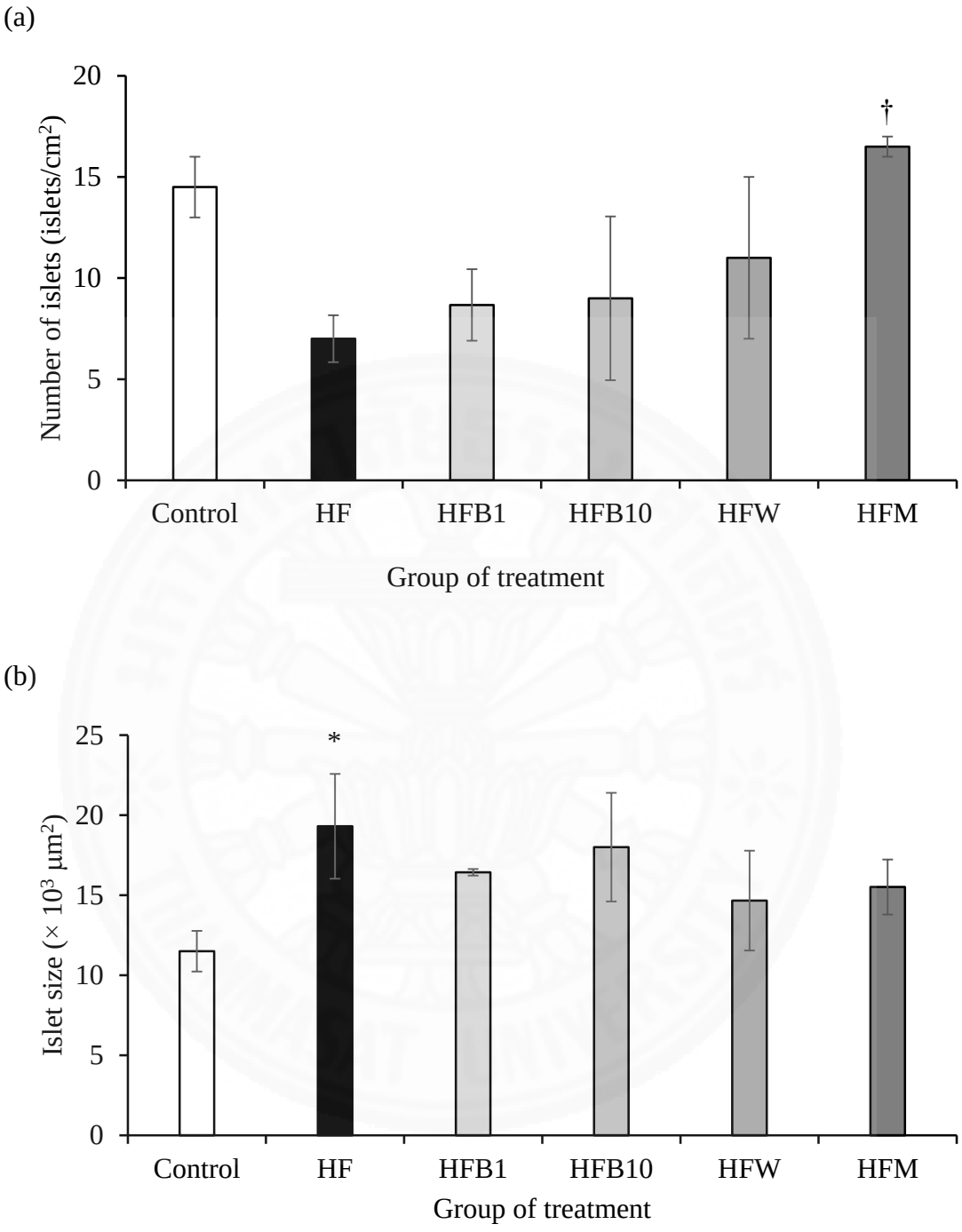


Figure 4.9 Number of islets (a) and islets size (b) in the short-term group. Values are expressed as mean ± SEM (n = 3)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

(a) Magnification ×200 (b) Magnification ×400

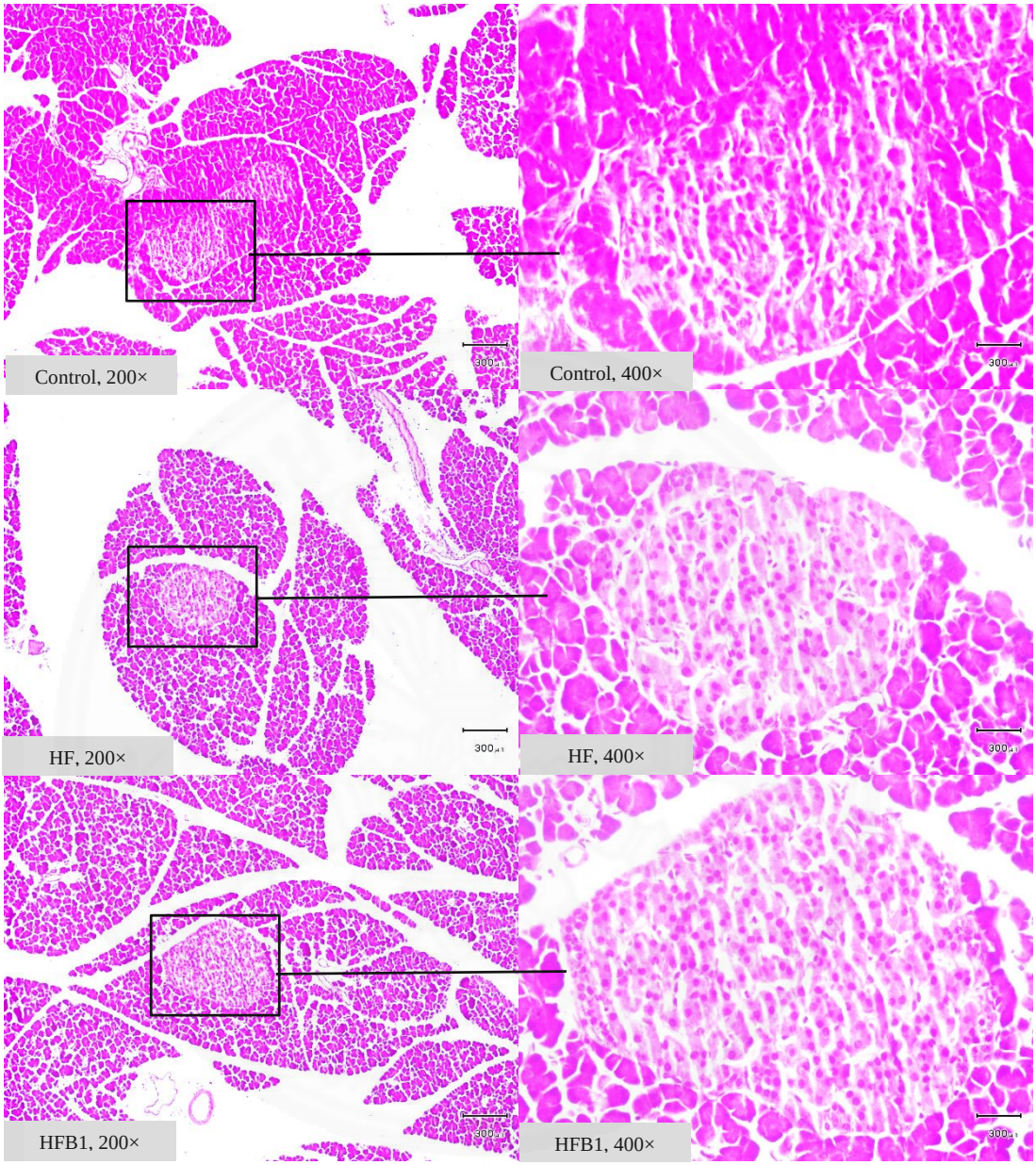


Figure 4.10 Histology of pancreatic islets of Langerhans (H & E staining, magnification 200× (a), 400× (b), scale bar = 100 μm) of intermediate-term groups (n = 3).

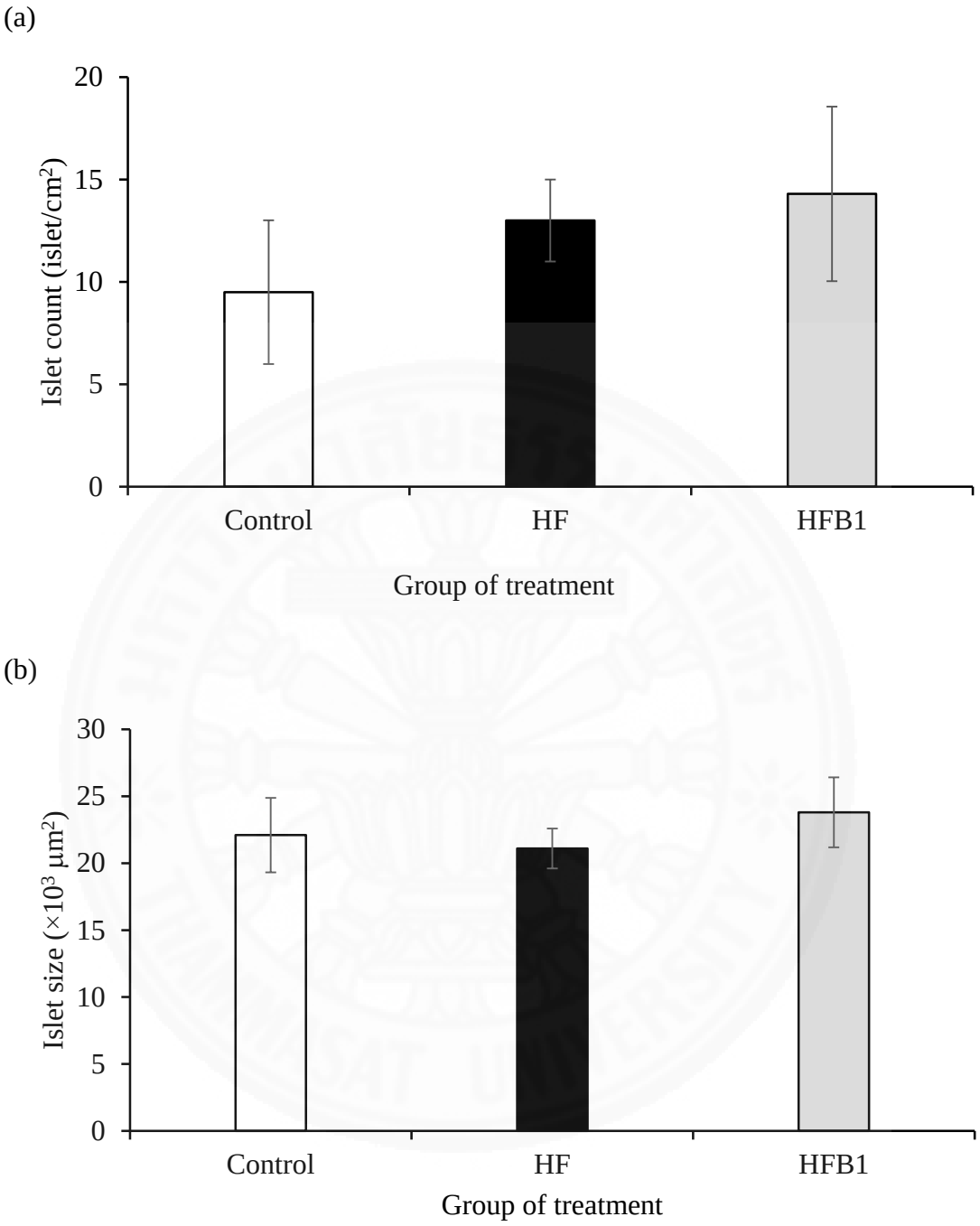


Figure 4.11 Number of islets (a) and islets size (b) in the intermediate-term group. Values are expressed as mean ± SEM (n = 3)

4.9.4 Effects of BWE on pancreatic senescence

4.9.4.1 Short-term experiment

The SA-β-gal staining in islets was shown in Figure 4.12. The density of SA-β-gal staining was significantly higher ($p < 0.05$) in the HF group than in the control group (Figure 4.14a). In the HFW and HFM groups, the density of SA-β-gal staining was significantly lower ($p < 0.05$) than in the HF group (Figure 4.14a). There was a trend toward a decrease in these densities in HFB1 and HFB10 rats, but the differences were not significant (Figure 4.14a).

4.9.4.2 Intermediate-term experiment

The SA-β-gal staining in islets is shown in Figure 4.13. Although, the density of SA-β-gal staining were no differences ($p > 0.05$) in all groups, there was a trend toward a decrease in this density in HFB1–treated rats (Figure 4.14b).

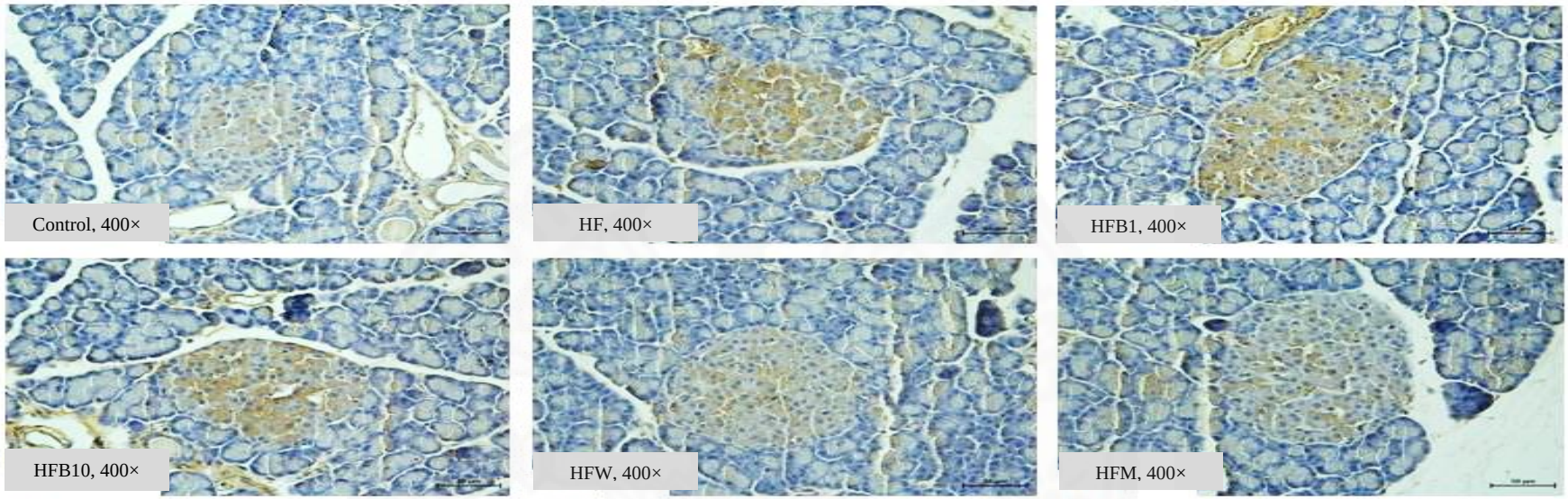


Figure 4.12 SA-β-gal staining in islets (magnification 400×, scale bar = 50 μm) in pancreas of the short-term groups (n=3).

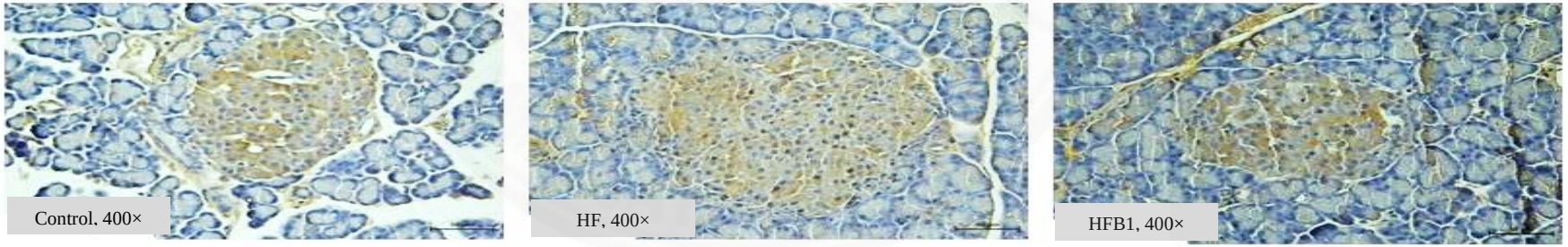
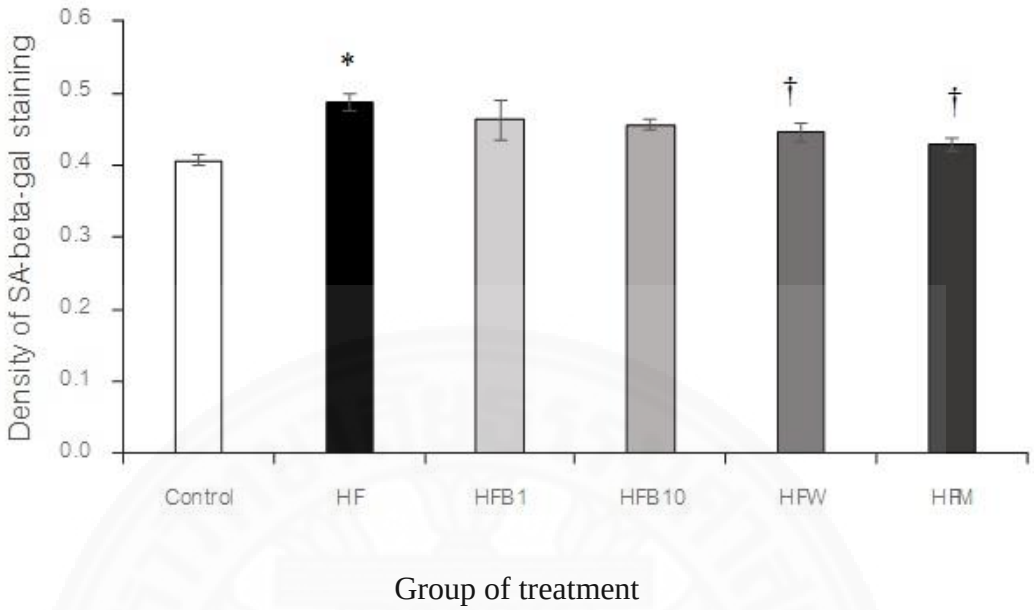


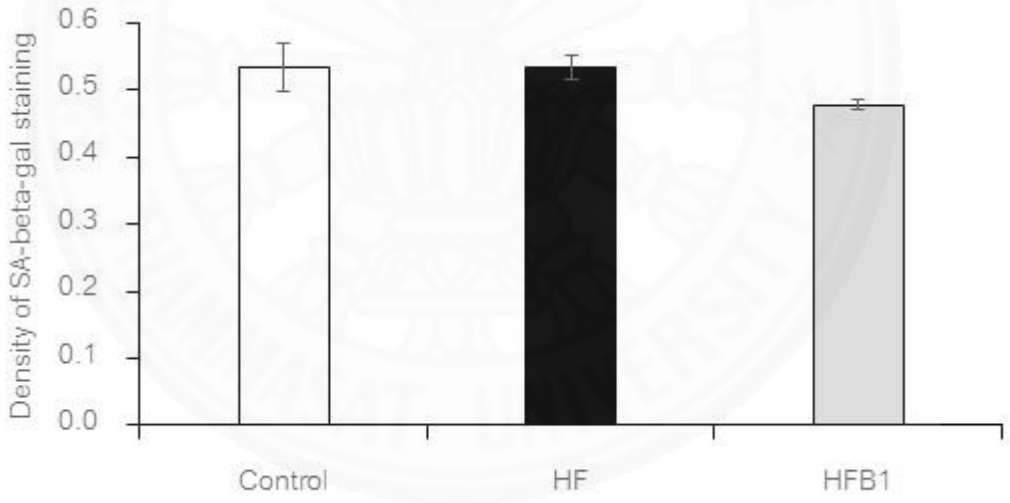
Figure 4.13 SA-β-gal staining in islets (magnification 400×, scale bar = 50 μm) in pancreas of the intermediate-term group (n=3).

(a)



Group of treatment

(b)



Group of treatment

Figure 4.14 Density of SA-β-gal staining in pancreas of the short-term group (a) and intermediate-term group (b). Values are expressed as mean ± SEM (n = 3).

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.10 Effects of BWE on pancreatic gene expression

4.10.1 NF-κB p65 gene expression

4.10.1.1 Short-term experiment

The effect of BWE on pancreatic NF-κB p65 expression of the short-term experimental groups was shown in Figure 4.15. Although there was no difference in the pancreatic NF-κB p65 mRNA expressions between the control and HF groups, the consumption of high-fat diet showed a tendency to increase in the mRNA levels when compared to the control group (Figure 4.15). Treatment with both BWE and metformin resulted in lower NF-κB p65 mRNA levels compared to the HF group. However, only the HFW group showed a significant up-regulation ($p < 0.05$) in the NF-κB p65 mRNA levels when compared to the control group (Figure 4.15).

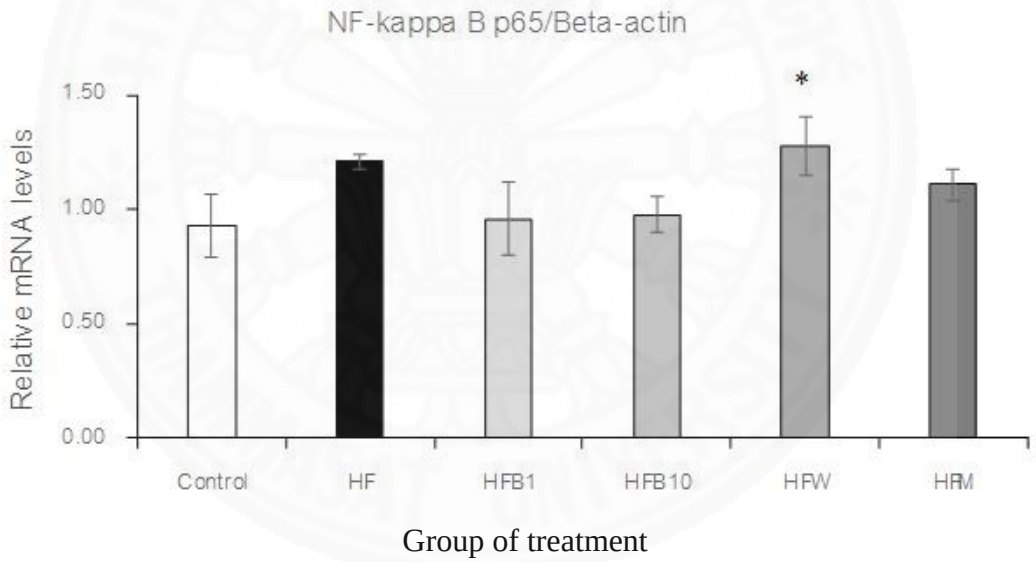


Figure 4.15 Pancreatic NF-κB p65 mRNA expression of the short-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control

4.10.1.2 Intermediate-term experiment

As shown in Figure 4.16, the expression levels of NF-κB p65 mRNA in the HF group were higher than in the control group. HFB1 group had significantly decreased NF-κB p65 mRNA levels when compared to the HF groups.

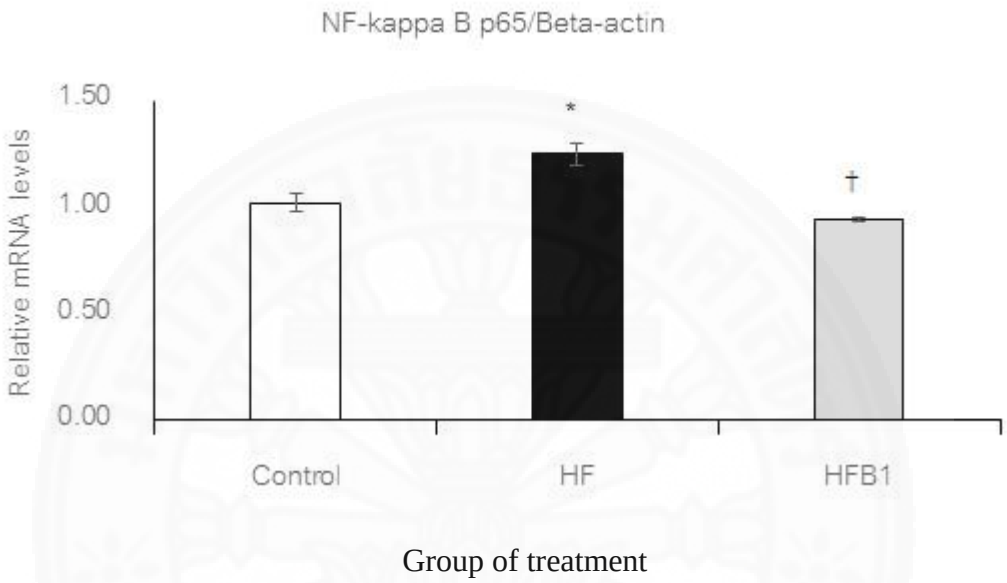


Figure 4.16 Pancreatic NF-κB p65 mRNA expression of the intermediate-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.10.2 IRS-2 gene expression

4.10.2.1 Short-term experiment

The HF and HFM groups showed a significant decrease in the IRS-2 mRNA level ($p < 0.05$) compared to the control group (Figure 4.17). The HFB1, HFB10, and HFW groups showed a significant increase in the IRS-2 mRNA level ($p < 0.05$) compared with the HF group (Figure 4.17).

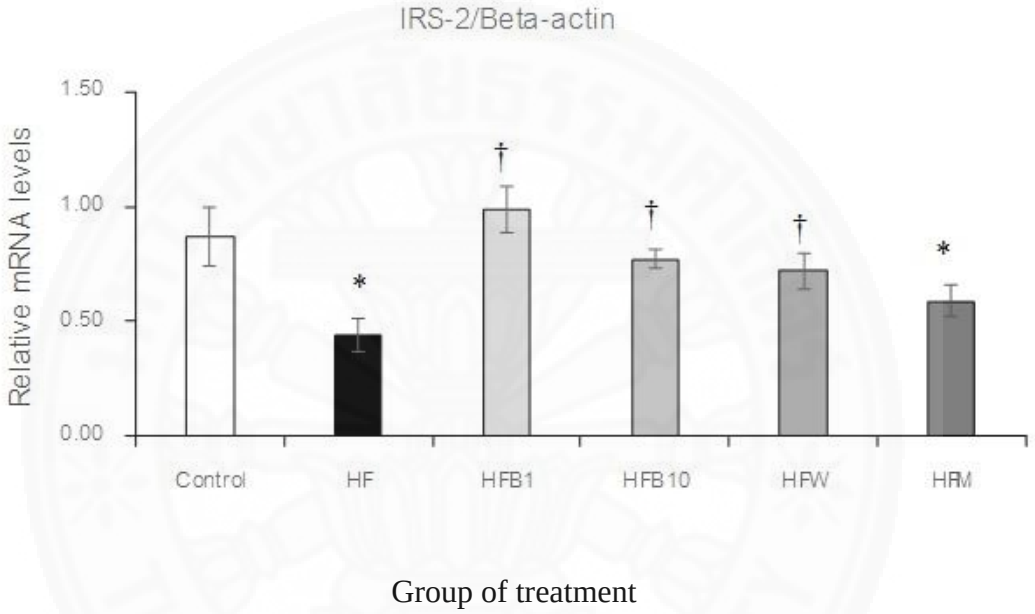


Figure 4.17 Pancreatic IRS-2 mRNA expression of the short-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.10.2.2 Intermediate-term experiment

As shown in Figure 4.18, the expression levels of IRS-2 mRNA were significantly higher in the HF group than in the control group. HFB1 group has significantly decreased IRS-2 mRNA levels when compared to the HF and control groups.

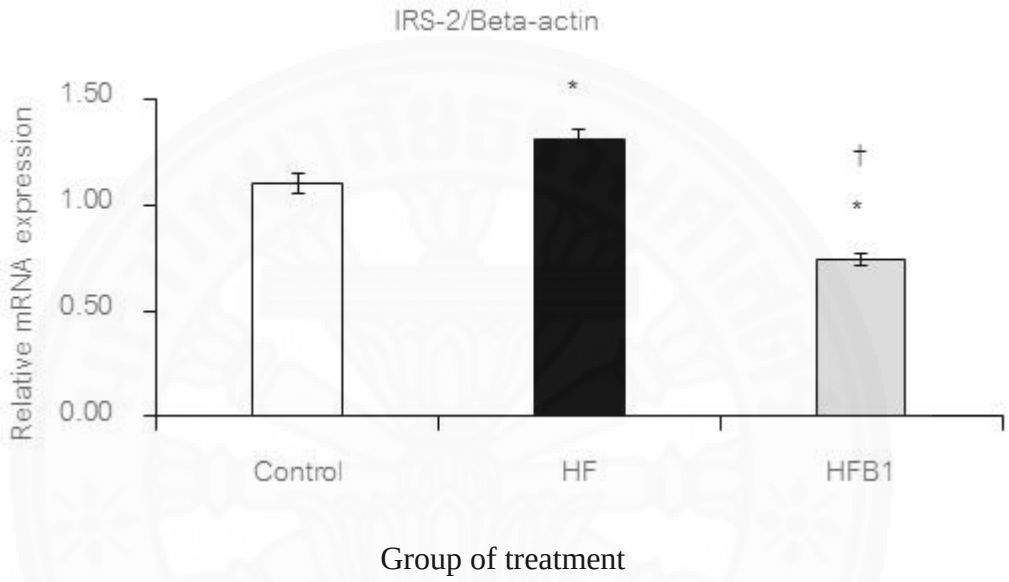


Figure 4.18 Pancreatic IRS-2 mRNA expression of the intermediate-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.10.3 GLUT-2 gene expression

4.10.3.1 Short-term experiment

The HF, HFB1, HFB10 and HFW groups showed a significant decrease in the GLUT-2 mRNA level ($p < 0.05$) compared with the control group (Figure 4.19). The HFW and HFM groups showed a significant increase in the GLUT-2 mRNA level ($p < 0.05$) compared to the HF group (Figure 4.19).

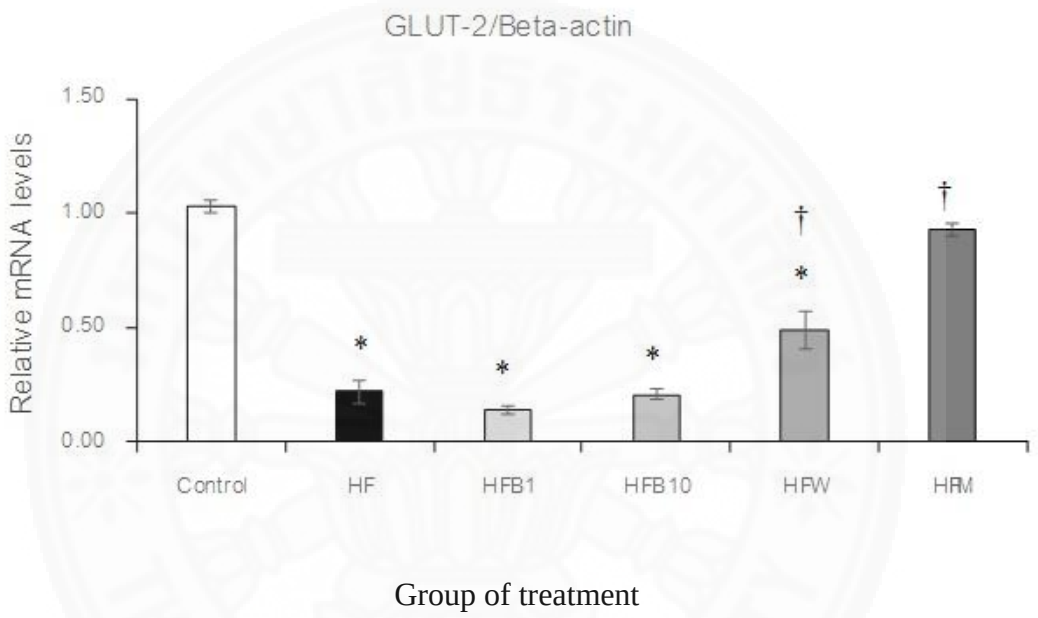


Figure 4.19 Pancreatic GLUT-2 mRNA expression of the short-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.10.3.2 Intermediate-term experiment

As shown in Figure 4.20, the expression levels of GLUT-2 mRNA in the HF group were significantly higher than in the control group. HFB1 group has significantly decreased GLUT-2 mRNA levels compared to the HF and control groups.

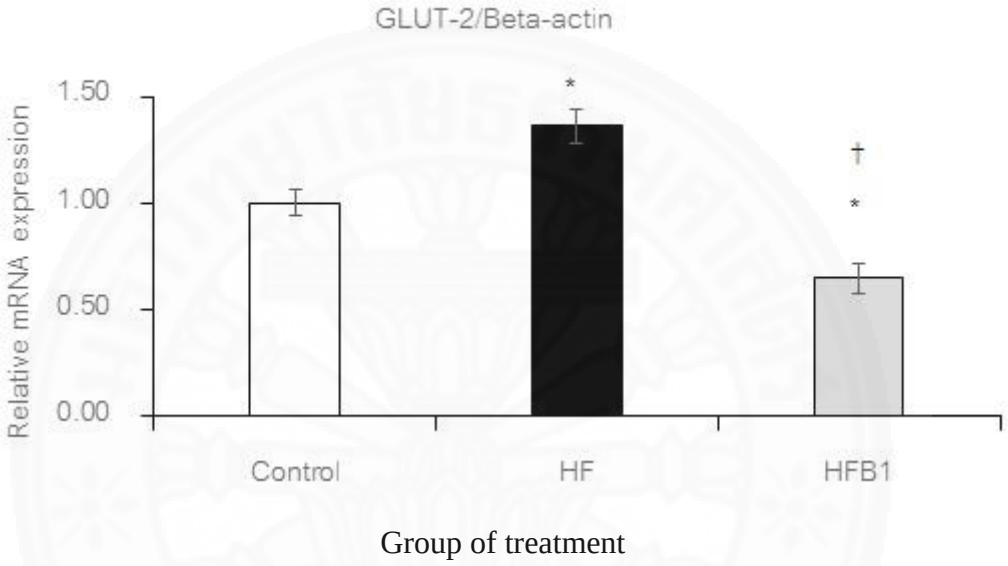


Figure 4.20 Pancreatic GLUT-2 mRNA expression of the intermediate-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

4.10.4 GK gene expression

4.10.4.1 Short-term experiment

The GK mRNA expressions in HF, HFB1, HFB10, HFW and HFM groups were significantly lower ($p < 0.05$) than the control group. The GK mRNA expressions in HFB1, HFB10, HFW and HFM were not significantly different from HF group (Figure 4.21).

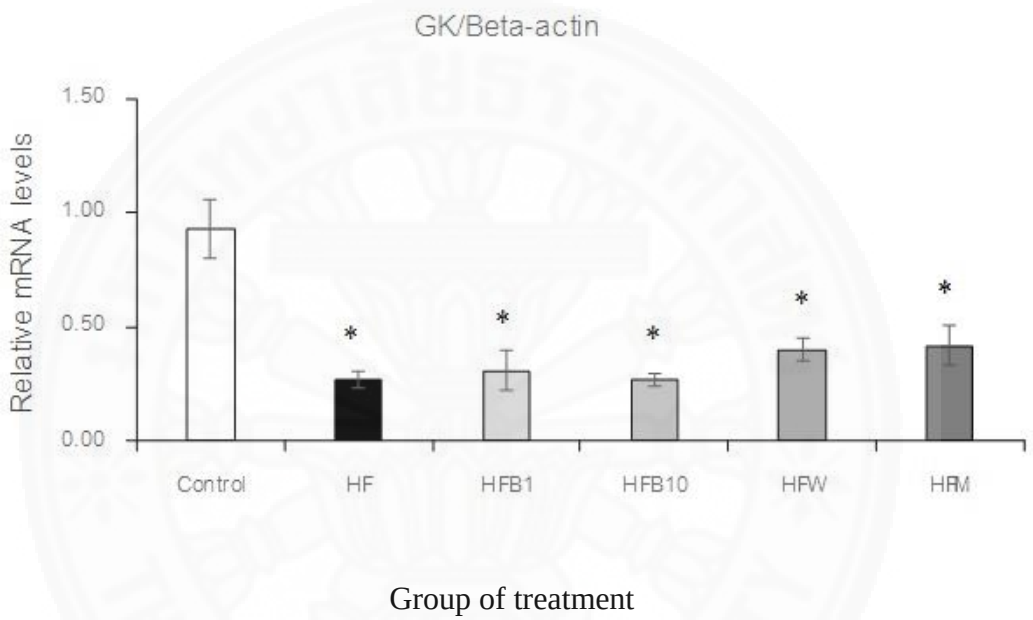


Figure 4.21 Pancreatic GK mRNA expression of the short-term groups. Values are expressed as mean ± SEM (n = 6)

* $p < 0.05$ vs. control

4.10.4.2 Intermediate-term experiment

As shown in Figure 4.22, the expression levels of GK mRNA in the HF group were significantly higher than in the control group. HFB1 group has significantly decreased GK mRNA levels when compared to the HF and control groups.

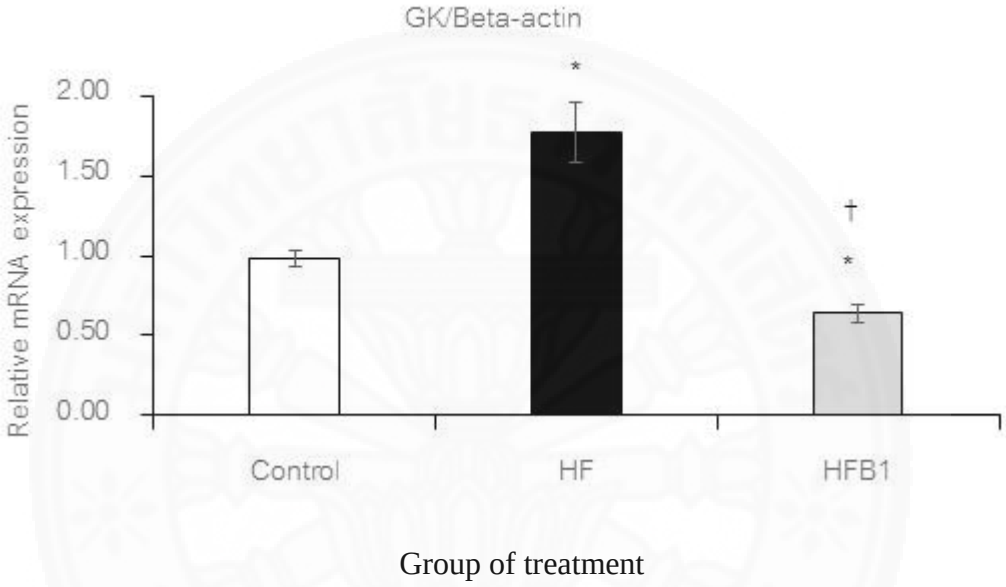


Figure 4.22 Pancreatic GK mRNA expression of the intermediate-term groups. Values are expressed as mean ± SEM (n = 6)

***** $p < 0.05$ vs. control, † $p < 0.05$ vs. HF

Table 4.14
Summary of results

Parameters	Short-term				Intermediate-term
	HFB1	HFB10	HFW	Met	HFB1
Food intake (g/day)	-	-	-	-	-
Energy intake (kcal/day)	-	-	-	-	-
Initial BW (g)	-	-	-	-	-
Final BW (g)	-	-	-	-	-
BW gain (%)	-	-	-	-	-
Pancreas (g/100 g BW)	-	-	-	↑	-
Abdominal fat (g/100 g BW)	↓	↓	-	↓	-
Epididymal fat (g/100 g BW)	-	↓	-	-	-
Adipocyte size (μm^2)	↓	↓	↓	↓	↓
Islet size (μm^2)	-	-	-	-	-
Islet count (islets/cm^2)	-	-	-	↑	-
Total cholesterol (mg/dL)	↓	-	-	↓	-
LDL-cholesterol (mg/dL)	↓	-	-	-	-
HDL-cholesterol (mg/dL)	↑	-	-	-	-
Triglyceride (mg/dL)	-	↓	-	↓	-
AST (U/L)	-	-	-	-	-
ALT (U/L)	-	-	-	-	-
BUN (mg/dL)	-	-	-	-	-
Creatinine (mg/dL)	-	-	-	-	-
Fasting blood glucose (mg/dL)	-	-	-	-	-
AUC-G (mg/dL.minutes)	-	-	-	-	-
Fasting serum insulin (mU/L)	-	-	-	-	-
OGTT (2 h)	-	↓	-	↓	-
SA beta-galactosidase	-	-	↓	↓	-
NF-kappa B p65	-	-	-	-	↓
IRS-2	↑	↑	↑	-	↓
GLUT-2	-	-	↑	↑	↓
GK	-	-	-	-	↓

All arrows indicating significance compared to high-fat diet. ↑, increased; ↓, decreased; -, no change; HFB1, low dose of BWE, HFB10, high dose of BWE; Met, metformin.

CHAPTER 5
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

The present study demonstrates the effects of BWE on metabolic risk factors and pancreatic abnormalities, including impaired insulin signaling, impaired glucose sensing and islet cell senescence, in a high-fat diet-induced metabolic syndrome in rats. The major findings of this study are:

- All BWE treatments can attenuate abnormal obesity and dyslipidemia
- All BWE treatments have no effect on glucose homeostatic parameters
- Short-term treatment with BWE can up-regulate pancreatic IRS-2 gene expression
- Short-term treatment with BWE was more effective than intermediate-term treatment in lowering metabolic and pancreatic abnormalities
- Intermediate-term treatment with BWE can cause abnormal conditions such as increased AUC of OGTT and down-regulation of IRS-2 gene

5.1 Effects of BWE on metabolic risk factors

As described, metabolic syndrome is a clustering of metabolic risk factors, including abdominal obesity, dyslipidemia, hyperglycemia, glucose intolerance and insulin resistance and is a major phenotype contributing to the diabetogenic state (Pittas, Joseph & Greenberg, 2004). The reduction of these metabolic risk factors is necessary for the prevention of DM. Overproduction of cytokines, such as TNF-α and IL-6, and FFA in adipose tissue can induce insulin resistance, in adipose tissue itself, as well as in liver and muscle (Thévenod, 2008) and lead to T2DM (Galic et al., 2010). In present study, adipocyte size were measured and abdominal and epididymal fat pads were weighted. The measurement of these adipose tissues has been previously used as a measure of body fat (de Freitas Mathias et al., 2007).

At four and sixteen weeks, the HF fed rats gained more weight compared to the standard chow-fed rats, which suggests that the high-fat diet have an effect on weight gain and obesity. With an increase in body weight, the abdominal fat tissue

weight and adipocyte size of the high-fat diet-fed rats were significantly increased compared with the control rats. The obese rats also exhibited dyslipidemia as indicated by increased levels of total cholesterol, LDL-C and TG in serum compared with the control rats. In comparison with the HF group, abdominal fat weight, adipocyte size, and serum total cholesterol and LDL-C levels were decreased, whereas serum HDL-C levels were increased in the short-term HFB1 group. Although there were no significant differences in serum total cholesterol, LDL-C and HDL-C levels between the control and HFB10 groups, short-term HFB10 group showed a significant reduction in abdominal fat weight, adipocyte size and serum TG levels when compared with the HF group. The same trends were observed for WWE and metformin treatments. Consistently, intermediate-term treatment with BWE markedly decreased adipocyte size. All high-fat diet-fed rats consumed more calories than the control diet-fed rats. Thus, the mechanism of anti-obesity effect may be mediated via the suppression of adipocyte hypertrophy, thereby reducing abdominal obesity. However, intermediate-term treatment with BWE could not decrease total cholesterol and LDL-C levels in serum. These results indicated that the short-term treatment with BWE is more effective than the intermediate-term treatment with BWE in regulating the dyslipidemia of animals under diet-induced obesity condition.

In addition to the abnormal lipid metabolism, the abnormal glucose homeostasis, such as hyperglycemia, glucose intolerance and insulin resistance, has been also observed in patients and animals with metabolic syndrome (Singla, Bardoloi & Parkash, 2010). At four weeks, although there was no difference in the serum levels of insulin among the groups, all high-fat diet-fed rats developed hyperglycemia and impaired OGTT compared with the controls. At sixteen weeks, there was a trend for AUC to be slightly higher in the obese rats when compared to the normal rats. Oral glucose administration was favoured as it obtains tissue response to glucose uptake particularly in muscle then liver and fat. Interference that occur as a result of gastrointestinal tract glucose uptake is reduced (DeFronzo, 2004). Our observations are concordant with those of Tanoue and colleagues (2011). Interestingly, both high-dose BWE and metformin treatments significantly protected impaired OGTT at 120 and 150 min. Moreover, there was a downward trend in blood glucose levels in control, low-dose BWE and WWE treatments, especially at 120–150 min after

glucose loading. However, treatments with BWE and WWE had no effect on the fasting blood glucose levels, fasting insulin levels and AUC in high-fat diet-fed rats. Unexpectedly, the intermediate-term HFB1 group had also significantly increased AUC, whereas the HF group did not have any significant changes compared with the control group. Unlike present results, previous study showed WWE treatment (125 mg/kg) significantly decreased the plasma glucose levels in diabetic rats (Peungvicha et al., 1998). Treatment with low dose of WWE (17 mg/kg) is therefore not sufficient to improve the abnormal glucose homeostasis of obese rats. Collectively, the findings of this study suggest that BWE has a tendency to improve the abnormal lipid metabolism (abdominal obesity and dyslipidemia) in a rat model of metabolic syndrome. Moreover, this study also suggested that treatment with BWE may not be appropriate for glucose homeostasis in the intermediate-term study and it may, therefore, not be suitable for intermediate-term use. It is possible that intermediate-term treatment with BWE inhibits expression of pancreatic insulin signaling and glucose-sensing genes, which may lead to glucose intolerance, as described below.

5.2 Effects of BWE on pancreatic abnormalities

5.2.1 Effects of BWE on the expression of insulin signaling and glucose-sensing genes

Consistent with previous studies, rats fed the high-fat diet alone in the short-term study developed impairments in insulin signaling and glucose sensing in the pancreases, which were characterized by the down-regulated expression of IRS-2, GLUT-2 and GK compared with rats fed a standard chow diet (Reimer & Ahren, 2002; Gremlich, Bonny, Waeber, & Thorens, 1997; Park, Hong, Lee, & Sung, 2007). Down-regulation of IRS-2 gene and impaired glucose-sensing pathways of β-cells lead to impaired GSIS, thus exacerbating the abnormalities of glucose metabolism (Assmann et al., 2009; Ahrén, 2009). The BWE and WWE treatments could up-regulate IRS-2 gene in the pancreases of rats fed a high-fat diet, suggesting that BWE and WWE treatments might prevent the impairment of insulin signaling in the pancreas. However, only the WWE-fed rats showed a significant increase in the

pancreatic GLUT-2 mRNA level. These results indicated that WWE may be the bioactive composition in the BWE formula for improving insulin signaling and glucose-sensing pathways. However, BWE treatment did not alter serum insulin levels, suggesting that the protective effect of BWE is not associated with a change in insulin secretion. Thus, improvement of insulin signaling and glucose sensing by BWE treatment may be a result of increased insulin sensitivity. In contrast to the short-term study, intermediate-term treatment with BWE notably suppressed pancreatic IRS-2, GLUT-2 and GK expressions. These suppressions might lead to the loss of insulin signaling and glucose-sensing pathways in the pancreas. Thus, BWE is not recommended for intermediate-term treatment of pancreatic abnormalities.

5.2.2 Effects of BWE on the pancreatic histology

The rats fed only with the high-fat diet showed signs of ectopic fat accumulation in the acinar cells, as determined by vacuoles. In agreement with this feature, studies in obese Zucker diabetic fatty rodents have shown that fat vacuoles were observed in the acinar cells before the onset of hyperglycemia (Lee et al., 2010). Recently, a study by Matsuda et al. also suggested that fat accumulation in the acinar cells was likely associated with the progression of pancreatic injury (Matsuda et al., 2014). Compared to the HF group, BWE, WWE and metformin could reduce the intracytoplasmic vacuoles in the acinar cells. Thus, these findings suggest that BWE and WWE may protect acinar cells from lipotoxicity. As mentioned above, BWE and WWE treatments could reduce hyperlipidemia and abdominal fat accumulation, which may be beneficial in the adjustment of lipid metabolism in the pancreas. Additional studies are necessary to fully investigate these protective effects of BWE. The rats fed only with the high-fat diet also showed decreased pancreatic mass, decreased islet number, and increased islet size, suggesting that these abnormalities may produce an abnormal glucose homeostasis in animals. Similar results were obtained in experimental animals fed a high-energy diet (Couturier et al., 2010; Sone & Kagawa, 2005).

5.2.3 Effects of BWE on pancreatic senescence

The pathogenesis of pancreatic senescence is associated with the increases in islet cell proliferation, oxidative stress and inflammation (Sone & Kagawa, 2005). One important pathway that induces cellular senescence is the up-regulation of NF-κB (Tilstra, Robinson, Wang, Gregg, Clauson, & Reay, 2012). Consistent with a previous study, the present results showed that the increased levels of senescent marker were seen in the pancreas of the short-term HF group (Sone & Kagawa, 2005). These increases coincided with the elevation of the NF-κB p65 mRNA levels. Thus, the current data suggest that HF can induce pancreatic senescence due to up-regulation of NF-κB. However, intermediate-term treatment with high-fat diet had no effect on pancreatic senescence. Consistent with this results, high-fat diet could up-regulate the expression of IRS-2, GLUT-2 and GK. All these findings suggest that they may be a compensatory process that helps to prevent the pancreatic abnormalities. Short-term treatment with BWE had no effect on pancreatic senescence. The present results suggest that the inhibition of pancreatic senescence is not a direct effect of pancreatic protection by BWE treatment. Only the intermediate-term HFB1 group showed a significant decrease in the pancreatic NF-κB p65 mRNA levels, suggesting that short-term HFB1 might delay the onset of anti-inflammation process. Pancreatic senescence was improved after the treatment with WWE, suggesting that WWE might delay the onset of pancreatic abnormalities via the reduction of pancreatic senescence. The same trends were observed for metformin treatment.

5.3 Effects of BWE on liver and kidney function tests

There were no difference in AST and ALT among all groups of treatment. AST and ALT levels indicated that liver have normal function. AST coupled with an enzyme ALT measurement for liver function test. The ALT is used to indicate a more danger to the liver than AST. The liver is the largest source of ALT in the body, while AST generated mostly by the heart muscle. There was significant decrease ($p < 0.05$) serum ALT level in normal volunteers, who received 100 mg orally of BWE (Amorndoljai, Kietinun & Somparn, 2011). Serum BUN and creatinine levels were

not statistically significant difference in all groups of treatment. This study was suggested that BWE and WWE had no adverse effect on liver and kidney.

5.4 Time and dose of BWE and WWE and metformin

The present study used time length for 4 weeks and 16 weeks, because life periods of rat are equal to adolescent and adult human respectively (Sengupta, 2013). Metabolic syndrome is a major risk factor for T2DM, a disease more common among adolescent and adult human (Hadjiyannakis, 2005). Previous studies have demonstrated that male Sprague-Dawley rats fed a high-fat diet for 4 weeks develop abdominal obesity, dyslipidemia and prediabetic state, compared with rats fed a normal diet (Chakhonpunya et al., 2011; Kaendee et al., 2009). In the previous study, rats fed a high-fat diet for 16 weeks also develop obesity, hyperlipidemia, hyperglycemia and glucose intolerance (Lecomte et al., 2015). Thus, these models were chosen for the present study. Dose of BWE 41.3 mg/kg BW and dose of WWE 17 mg/kg BW from translated dose of adult human assigned by the National List of Medicine, 2013. High dose of BWE 413 mg/kg BW (ten folds of low dose) adapted from Tosa Benjakul. High dose of BWE is not use in intermediate-term treatment because it may cause adverse effect.

Unlike present data, previous study showed treatment with metformin (100 and 300 mg/kg BW daily) significantly reduced body weight, insulin resistance and hypertriglyceridemia in rats (Chen et al., 2004; Yan et al., 2015). It is possible that treatment with low dose of metformin (19.5 mg/kg BW daily) is not sufficient to affect the metabolic dysfunctions of obese rats.

5.5 Conclusion and recommendations

In conclusion, the present study indicated that BWE may exert adaptogenic activities on lipid homeostasis in a metabolic syndrome rat model. BWE may also exert protective activities on high-fat diet-induced pancreatic abnormalities. There are probably due to the activation of IRS2 gene expression and the reduction of vacuole accumulation in the pancreas. Short-term treatment with BWE was more

effective than intermediate-term treatment in inhibiting dyslipidemia and pancreatic abnormalities. Lower dose (41.3 mg/kg) of BWE is much more effective in improving the metabolic and pancreatic abnormalities than its respective higher dose (413 mg/kg) in rats with metabolic syndrome. Thus, the present findings provided evidence that short-term BWE treatment, especially low-dose treatment, may be useful as an adaptogenic formula for the modulation of the impaired lipid metabolism and pancreatic abnormalities. However, BWE treatment is not sufficient to improve the abnormal glucose homeostasis of rats with metabolic syndrome. Furthermore, this study suggested that treatment with BWE may not be appropriate for pancreases and glucoregulation of rats in the intermediate-term study and it may, therefore, not be suitable for intermediate-term use. The conclusion of the results responsible for the short-term and intermediate-term effects of BWE on metabolic syndrome are summarized in figure 5.1 and 5.2. Although BWE may protect against pancreatic changes, further studies are required to examine the pharmacokinetics and molecular basis for the pharmacological activity of BWE on metabolic dysfunctions and pancreas in the management of diabetes mellitus. Moreover, the results suggested that the contribution of antioxidant activity could be due to the effect of phenolic compounds in BWE and WWE. These properties of BWE and WWE may contribute to the improvement of metabolic dysfunctions and pancreatic abnormalities in high-fat diet-fed rats. However, the bioactive compounds in BWE and WWE are still unknown. Further studies are needed to illuminate the bioactive constituents in BWE and WWE. The effect of Benjakul alcoholic extract on pancreas in rats fed a high-fat diet may have to studying in the future.

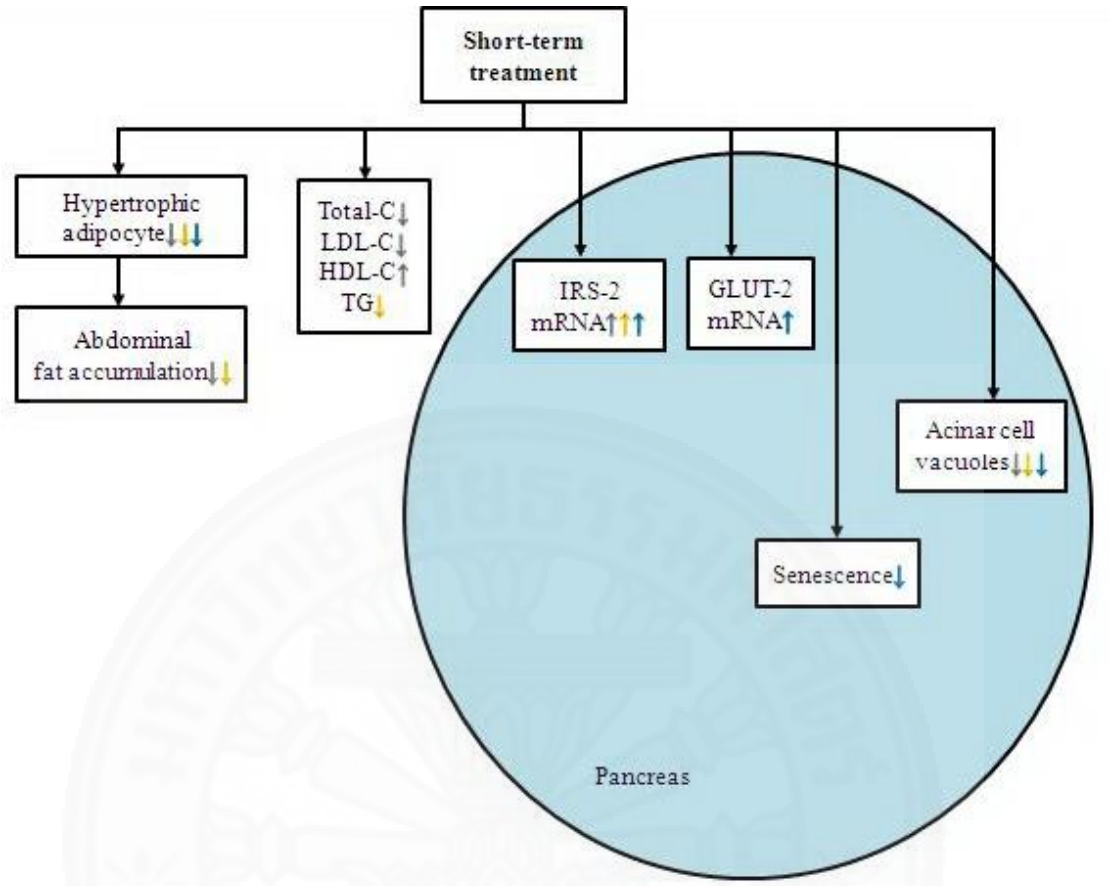


Figure 5.1 Conclusion of the results for the short-term effects of BWE on metabolic syndrome.

↑, increased; ↓, decreased; gray arrow, BWE (41.3 mg/kg BW/day); yellow arrow, BWE (413 mg/kg BW/day); blue arrow, WWE (17 mg/kg BW/day)

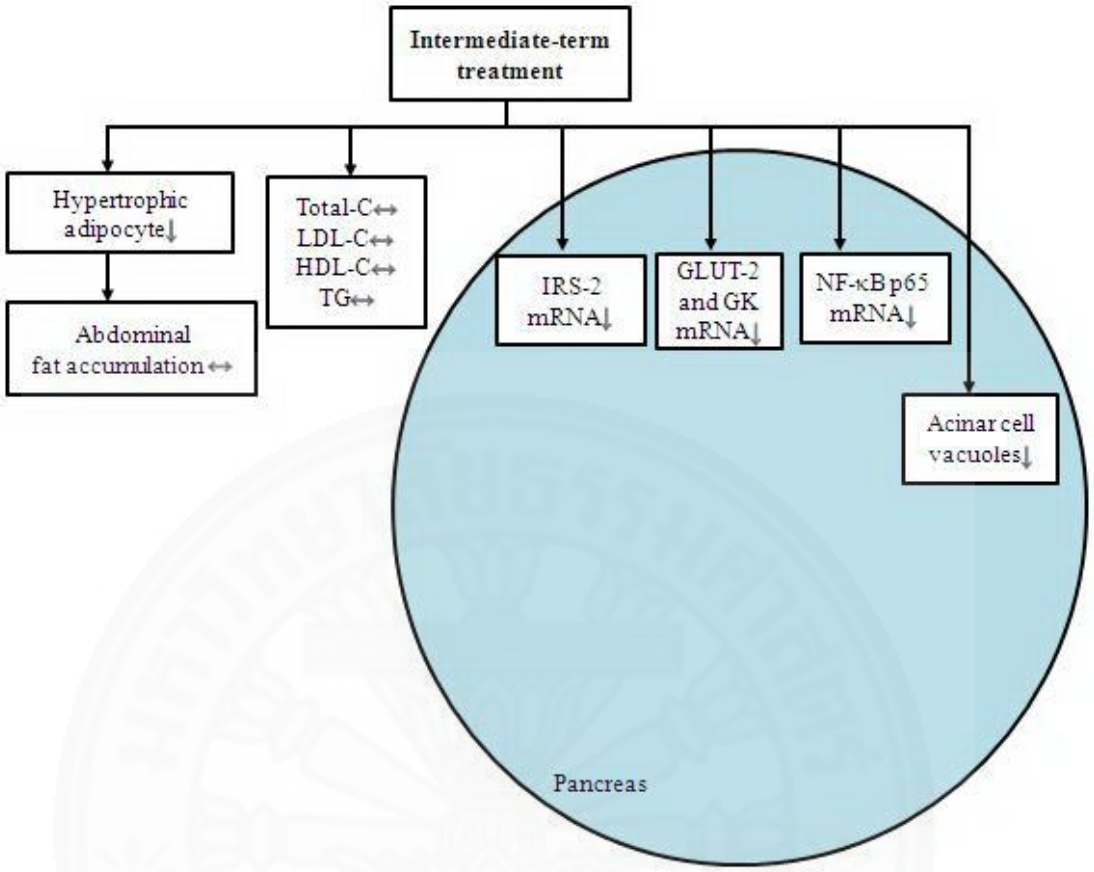


Figure 5.2 Conclusion of the results for the intermediate-term effects of BWE on metabolic syndrome.

↓, decreased; ↔, no change; gray arrow, BWE (41.3 mg/kg BW/day)

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APPENDICES

APPENDIX A

NATIONAL LIST OF ESSENTIAL MEDICINES 2012

ประกาศคณะกรรมการพัฒนาระบบยาแห่งชาติ
เรื่อง บัญชียาหลักแห่งชาติ
พ.ศ. ๒๕๕๕

อาศัยอำนาจตามความในข้อ ๘(๔) แห่งระเบียบสำนักนายกรัฐมนตรีว่าด้วยคณะกรรมการพัฒนาระบบยาแห่งชาติ พ.ศ.๒๕๕๑ ได้กำหนดให้คณะกรรมการพัฒนาระบบยาแห่งชาติ มีอำนาจหน้าที่ในการจัดทำบัญชียาหลักแห่งชาติ เพื่อให้บัญชียาหลักแห่งชาติมีการปรับปรุงแก้ไข ตามสภาพของปัญหาสุขภาพ วิทยาการ และข้อมูลเกี่ยวกับยาที่เปลี่ยนแปลงไปอย่างต่อเนื่องทันสถานการณ์

คณะกรรมการพัฒนาระบบยาแห่งชาติ จึงออกประกาศไว้ดังต่อไปนี้

ข้อ ๑ ให้ยกเลิก

(๑) ประกาศคณะกรรมการแห่งชาติด้านยา เรื่อง บัญชียาหลักแห่งชาติ พ.ศ.๒๕๕๑ ลงวันที่ ๒๓ มกราคม พ.ศ. ๒๕๕๑

(๒) ประกาศคณะกรรมการแห่งชาติด้านยา เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๒) พ.ศ.๒๕๕๑ ลงวันที่ ๓ กุมภาพันธ์ พ.ศ. ๒๕๕๑

(๓) ประกาศคณะกรรมการแห่งชาติด้านยา เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๓) พ.ศ.๒๕๕๑ ลงวันที่ ๒๐ มิถุนายน พ.ศ. ๒๕๕๑

(๔) ประกาศคณะกรรมการแห่งชาติด้านยา เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๔) พ.ศ.๒๕๕๒ ลงวันที่ ๑๖ กุมภาพันธ์ พ.ศ. ๒๕๕๒

(๕) ประกาศคณะกรรมการแห่งชาติด้านยา เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๕) พ.ศ.๒๕๕๒ ลงวันที่ ๗ พฤษภาคม พ.ศ. ๒๕๕๒

(๖) ประกาศคณะกรรมการพัฒนาระบบยาแห่งชาติ เรื่อง บัญชียาหลักแห่งชาติ พ.ศ. ๒๕๕๓ ลงวันที่ ๓๓ ตุลาคม ๒๕๕๓

(๗) ประกาศคณะกรรมการพัฒนาระบบยาแห่งชาติ เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๒) พ.ศ.๒๕๕๓ ลงวันที่ ๑๕ ธันวาคม ๒๕๕๓

(๘) ประกาศคณะกรรมการพัฒนาระบบยาแห่งชาติ เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๓) พ.ศ.๒๕๕๔ ลงวันที่ ๙ พฤษภาคม ๒๕๕๔

(๙) ประกาศคณะกรรมการพัฒนาระบบยาแห่งชาติ เรื่อง บัญชียาหลักแห่งชาติ (ฉบับที่ ๔) พ.ศ.๒๕๕๔ ลงวันที่ ๒๕ พฤษภาคม ๒๕๕๔

ข้อ ๒ ให้ใช้รายการยาในบัญชีแนบท้ายประกาศฉบับนี้เป็นยาในบัญชียาหลักแห่งชาติ

ทั้งนี้ให้มีผลบังคับใช้ตั้งแต่บัดนี้เป็นต้นไป

ประกาศ ณ วันที่ ๒๔ มีนาคม พ.ศ. ๒๕๕๕

พลเอก
(ยุทธศักดิ์ ศศิประภา)
รองนายกรัฐมนตรี
ประธานกรรมการพัฒนาระบบยาแห่งชาติ

APPENDIX B
ORGANIC CERTIFICATION

กรมวิชาการเกษตร
กระทรวงเกษตรและสหกรณ์

ใบรับรองฉบับนี้ให้ไว้เพื่อแสดงว่า

นายดำรงศักดิ์ ชุมแสงพันธ์
99 หมู่ 1 ตำบลป่ายุบใน อำเภอวังจันทร์ จังหวัดระยอง

ได้การรับรองมาตรฐานการผลิตพืชอินทรีย์ พ.ศ. 2543
กรมวิชาการเกษตร

รหัสรับรอง TAS:2140

ขอบข่ายการรับรอง : แหล่งผลิตพืชอินทรีย์
สมุนไพร ผัก ผลไม้ (มังคุด เงาะ ทุเรียน) และนาข้าว พื้นที่ 150 ไร่

วันที่รับรอง 24 เดือน กันยายน พ.ศ. 2552 วันที่หมดอายุ 23 เดือน กันยายน พ.ศ. 2553

Organic Thailand
ผลิตภัณฑ์อินทรีย์

(นางสาวเสริมสุข สลักเพ็ชร์)
ผู้อำนวยการสำนักวิจัยและพัฒนาการเกษตรเขตที่ 6
ปฏิบัติราชการแทนอธิบดีกรมวิชาการเกษตร

กรมวิชาการเกษตร

สำนักวิจัยและพัฒนาการเกษตรเขตที่ 6 ต.ตะปอน อ.ขลุง จ.จันทบุรี 22190 โทร 039-397076 oard6@doa.in.th

APPENDIX C

BENJAKUL DRUG INSTRUCTION

ขนาดและวิธีใช้	รับประทานครั้งละ 1 กรัม ชงน้ำร้อนประมาณ 120 - 200 มิลลิลิตร ดื่มขณะยายังอุ่น วันละ 4 ครั้ง ก่อนอาหารและก่อนนอน
ข้อห้ามใช้	-
ข้อควรระวัง	- ไม่ควรรับประทานติดต่อกันเกิน 1 เดือน - หากใช้เกินจากขนาดที่แนะนำ อาจจะทำให้ท้องผูก - ควรระวังการใช้ยาในผู้ป่วยที่แพ้ละอองเกสรดอกไม้
อาการไม่พึงประสงค์	-
ข้อมูลเพิ่มเติม	-

(2) ยาตรีพิกัด ยาแคปซูล (รพ.)

สูตรตำรับ	ในผงยา 90 กรัม ประกอบด้วย 1. เนื้อลูกสมอไทย เนื้อลูกสมอพิเภก เนื้อลูกมะขามป้อม หนักสิ่งละ 10 กรัม 2. เหง้าขิงแห้ง พริกไทยล่อน ดอกดีปลี หนักสิ่งละ 10 กรัม 3. รากเจตมูลเพลิงแดง รากช้าพลู เถาสะค้าน หนักสิ่งละ 10 กรัม
ข้อบ่งใช้	ปรับสมดุลธาตุ
ขนาดและวิธีใช้	รับประทานครั้งละ 250 – 500 มิลลิกรัม วันละ 3 ครั้ง ก่อนอาหาร
ข้อห้ามใช้	ห้ามใช้ในหญิงตั้งครรภ์ ผู้ที่มีไข้
ข้อควรระวัง	- ไม่ควรใช้ยานี้ในฤดูร้อน ส่งผลให้ไฟธาตุกำเริบ - ควรระวังการใช้ร่วมกับยา phenytonin, propranolol, theophylline และ rifampicin เนื่องจากตำรับนี้มีพริกไทยและดอกดีปลีในปริมาณสูง
อาการไม่พึงประสงค์	-
ข้อมูลเพิ่มเติม	-

(3) ยาเบญจกูล ยาแคปซูล ยาผง ยาเม็ด ยาลูกกลอน ยาแคปซูล (รพ.) ยาชง (รพ.) ยาเม็ด (รพ.)

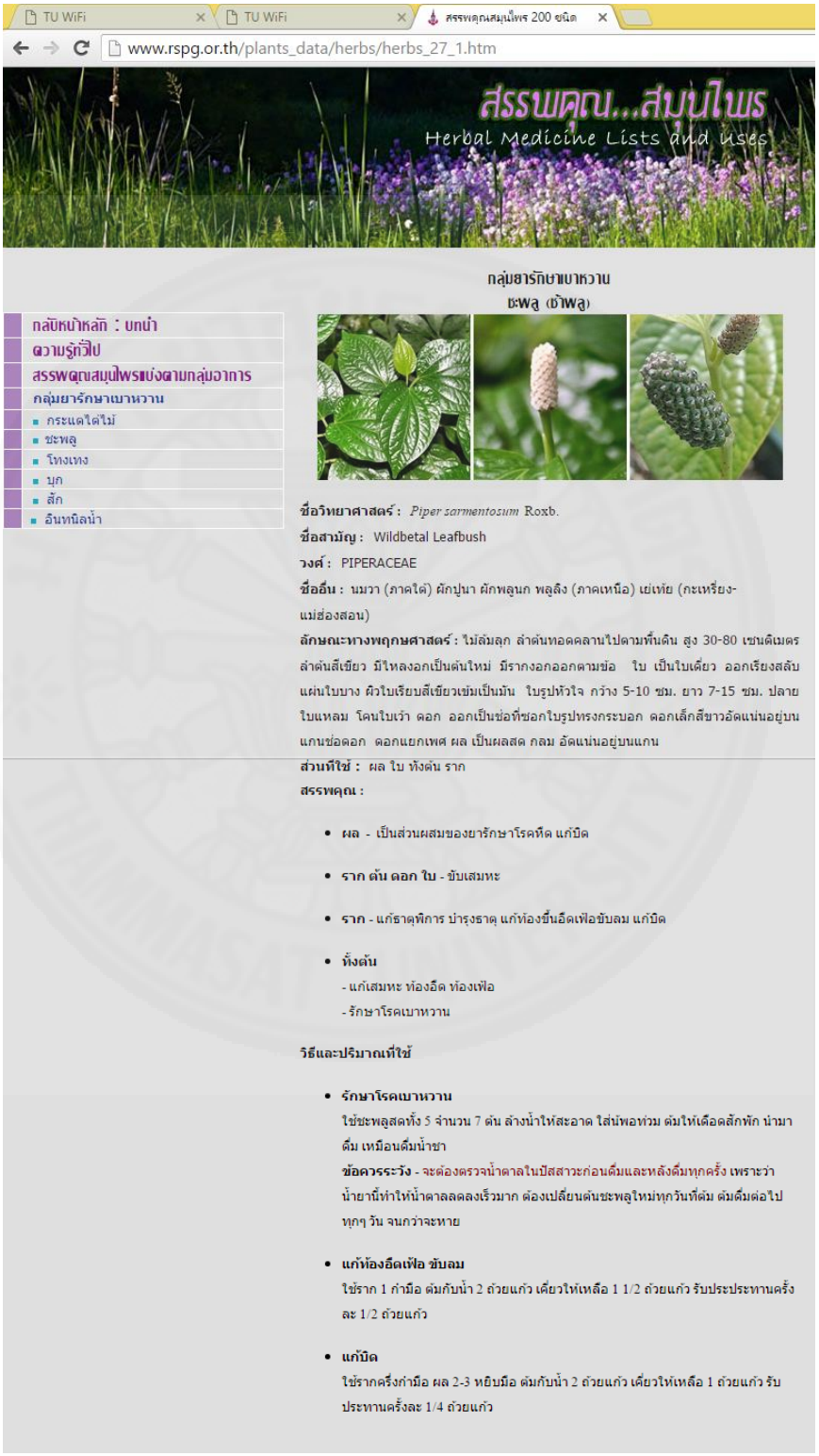
สูตรตำรับ	ในผงยา 100 กรัม ประกอบด้วย ดอกดีปลี รากช้าพลู เถาสะค้าน รากเจตมูลเพลิงแดง เหง้าขิงแห้ง หนักสิ่งละ 20 กรัม
ข้อบ่งใช้	บำรุงธาตุ แก้ธาตุให้ปกติ
ขนาดและวิธีใช้	**ชนิดชง** รับประทานครั้งละ 1.5 – 2 กรัม วันละ 3 ครั้ง หลังอาหาร **ชนิดผง** รับประทานครั้งละ 800 มิลลิกรัม – 1 กรัม วันละ 3 เวลา หลังอาหาร **ชนิดลูกกลอน ชนิดเม็ด และชนิดแคปซูล** รับประทานครั้งละ 800 มิลลิกรัม – 1 กรัม วันละ 3 ครั้ง หลังอาหาร
ข้อห้ามใช้	ห้ามใช้ในหญิงตั้งครรภ์ ผู้ที่มีไข้ และเด็กเล็ก
ข้อควรระวัง	- ไม่ควรใช้ยานี้ในฤดูร้อน จะส่งผลให้ไฟธาตุกำเริบ - ไม่ควรรับประทานติดต่อกันนานเกิน 7 วัน
อาการไม่พึงประสงค์	-
ข้อมูลเพิ่มเติม	-

(4) ยาปลูกไฟธาตุ ยาแคปซูล (รพ.) ยาลูกกลอน (รพ.)

สูตรตำรับ	ในผงยา 100 กรัม ประกอบด้วย

APPENDIX D

WILD BETAL LEAF BUSH FOR DIABETIC TREATMENT



Retrieved form http://rspg.or.th/plants_data/herbs/herbs_27_1.htm

APPENDIX E

THE ANIMAL ETHIC APPROVAL

เลขที่ AE 001/2015

ใบรับรองให้ดำเนินการวิจัยในสัตว์ทดลอง

คณะกรรมการจรรยาบรรณและติดตามผลโครงการวิจัย

การใช้สัตว์เพื่องานทางวิทยาศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ชื่อโครงการวิจัย

(ภาษาไทย) อาหารและ/ หรือสมุนไพรป้องกันเบาหวานและลดไขมันในเลือดสูง

(ภาษาอังกฤษ) Thai food and herbal medicine in preventing diabetes and dyslipidemia

ชื่อ-สกุล ผู้เสนอโครงการวิจัย รองศาสตราจารย์ ดร. นุชสิริ เลิศวุฒิโสภณ

หน่วยงานสังกัด (คณะ/กอง) คณะแพทยศาสตร์

(มหาวิทยาลัย/กรม) มหาวิทยาลัยธรรมศาสตร์

(กระทรวง) -

คณะกรรมการจรรยาบรรณและติดตามผลโครงการวิจัยการใช้สัตว์เพื่องานทางวิทยาศาสตร์ มหาวิทยาลัยธรรมศาสตร์ มีมติให้โครงการดังกล่าวสามารถดำเนินการวิจัยได้ โดยยึดหลักจรรยาบรรณการใช้สัตว์อย่างเคร่งครัด หากได้ตรวจสอบพบว่าผู้ดำเนินการวิจัยในโครงการนี้ได้ดำเนินการวิจัยผิดหลักจรรยาบรรณการใช้สัตว์ คณะกรรมการจรรยาบรรณและติดตามผลโครงการวิจัยการใช้สัตว์เพื่องานทางวิทยาศาสตร์ มหาวิทยาลัยธรรมศาสตร์ มีสิทธิเพิกถอนใบรับรองนี้

ลงนาม..... ลงนาม.....

(ผู้ช่วยศาสตราจารย์ ดร. ปริศนา ปิยะพันธุ์) (ศาสตราจารย์ นายแพทย์ก้องเกียรติ กุณฑ์กันทรากร)

ประธานคณะกรรมการจรรยาบรรณและติดตาม ผลโครงการวิจัยการใช้สัตว์เพื่องานทางวิทยาศาสตร์

รองคณบดีฝ่ายวิจัย

วัน/เดือน/ปี ๒๑ มกราคม ๒๕๕๘ วัน/เดือน/ปี ๒๗ มกราคม ๒๕๕๘

APPENDIX F
CHEMICAL REAGENTS

1. 1× PBST

- 10× Stocking PBS 100 mL
- Adjust to pH 7.2 and adjust to a final volume of 1,000 mL with DW
- Tween-20 0.1 mL

1× PBST can be used as the diluent buffer for the blocking serum and secondary antibody.

2. Hyaluronidase digest

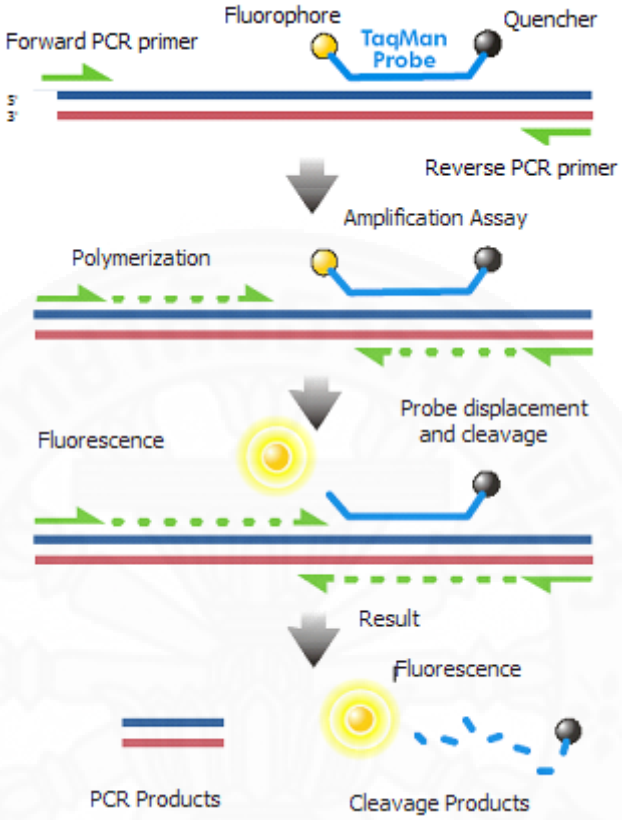
- 1× PBS 250 mL
- Hyaluronidase 25 mg

3. 3% H_2O_2

- Methanol 8 mL
- H_2O_2 0.24 mL

APPENDIX G

THE PRINCIPLE OF TaqMan REAL-TIME PCR



Retrieved form https://en.wikipedia.org/wiki/TaqMan#/media/File:Taqman.png

APPENDIX H

PROXIMATE ANALYSIS OF BENJAKUL

บริษัท เซ้าท์อีสต์เอเชี่ยนลาบอราทอรี่ย์ จำกัด (ซีล)
SOUTH EAST ASIAN LABORATORY LTD. (SEAL)

ORIGINAL
หน้า 1 ของ 1

รายงานผลการทดสอบ

เลขที่ใบรายงานผลการทดสอบ 1301838
หมายเลขทดสอบ : 1861/2013 วันที่ออกใบรายงานผล : 25 มีนาคม 2556

วันที่รับตัวอย่าง : 13 มีนาคม 2556 วันที่ทดสอบ : 14 มีนาคม 2556

ชื่อลูกค้า : คณะแพทย์ศาสตร์ มหาวิทยาลัยธรรมศาสตร์
ที่อยู่ : คณะแพทย์ศาสตร์ มหาวิทยาลัยธรรมศาสตร์ คลิกคณะแพทย์ศาสตร์ ชั้น 5 ห้อง 4508 อำเภอคลองหลวง จังหวัด ปทุมธานี

ชื่อและรหัสตัวอย่าง : BJ (Proximate analysis)
รายละเอียดของตัวอย่าง : ผงบดหยาบสีดำ บรรจุในถุงพลาสติก, น้ำหนัก 50 กรัม ต่อ ภาชนะบรรจุ

ผลการทดสอบ :

รายการทดสอบ	ผลทดสอบ	หน่วย	วิธีทดสอบ
พลังงาน	244	กิโลแคลอรี่/100 กรัม	AOAC International,1993 (Chapter6)
พลังงานจากไขมัน	2	กิโลแคลอรี่/100 กรัม	AOAC International,1993 (Chapter6)
คาร์โบไฮเดรต	46.32	กรัม/100 กรัม	AOAC International,1993 (Chapter1)
โปรตีน	14.20	กรัม/100 กรัม	Based on AOAC (2005) 979.09
ไขมัน	0.20	กรัม/100 กรัม	Based on AOAC(2005) 922.06
เถ้า	25.83	กรัม/100 กรัม	Based on AOAC (2005) 945.46
ความชื้น	13.45	กรัม/100 กรัม	AOAC (2005) 990.20

(นางสาวสุภาวดี วิจารณ์)
ผู้จัดการแผนกเคมี

(นางสาวธาริณี วิภูษนิน)
กรรมการผู้จัดการ

รายงานฉบับนี้รับรองเฉพาะตัวอย่างที่ทดสอบเท่านั้น
ห้ามคัดถ่ายใบรายงานผลการทดสอบแต่เพียงบางส่วนโดยไม่ได้รับอนุญาตจากห้องปฏิบัติการเป็นลายลักษณ์อักษร

FM-CS-2301 (130705)

256/1 ซอยสันนิบาตเทศบาล ถนนรัชดาภิเษก แขวงจันทรเกษม เขตจตุจักร กรุงเทพฯ 10900 โทร. 0-2939-1131-3, 0-2511-6113-5 โทรสาร 0-2512-3821, 0-2511-6114
256/1 Soi Sannibat-Tesaban, Ratchadapisek Road, Chatuchak, Bangkok 10900, Thailand. Tel. 0-2939-1131-3, 0-2511-6113-5 Fax: 0-2512-3821, 0-2511-6114
E-mail address : customerservices@seallab.co.th

APPENDIX I

PROXIMATE ANALYSIS OF WILD BETAL LEAF BUSH

บริษัท เซ้าท์อีสต์เอเชี่ยนลาบอราทอรี่ย์ จำกัด (ซีล)
SOUTH EAST ASIAN LABORATORY LTD. (SEAL)

ORIGINAL
หน้า 1 ของ 1

รายงานผลการทดสอบ

เลขที่ใบรายงานผลการทดสอบ 1301837
หมายเลขทดสอบ : 1860/2013 วันที่ออกใบรายงานผล : 25 มีนาคม 2556
วันที่รับตัวอย่าง : 13 มีนาคม 2556 วันที่ทดสอบ : 14 มีนาคม 2556
ชื่อลูกค้า : คณะแพทย์ศาสตร์ มหาวิทยาลัยธรรมศาสตร์
ที่อยู่ : คณะแพทย์ศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ตึกคณะแพทย์ศาสตร์ ชั้น 5 ห้อง 4508 อำเภอคลองหลวง จังหวัด ปทุมธานี
ชื่อและรหัสตัวอย่าง : CH (Proximate analysis)
รายละเอียดของตัวอย่าง : ผงบดหยาบสีเขียวบรรจุในถุงพลาสติก น้ำหนัก 50 กรัม ต่อภาชนะบรรจุ

ผลการทดสอบ :

รายการทดสอบ	ผลทดสอบ	หน่วย	วิธีทดสอบ
พลังงาน	264	กิโลแคลอรี่/100 กรัม	AOAC International,1993 (Chapter6)
พลังงานจากไขมัน	3	กิโลแคลอรี่/100 กรัม	AOAC International,1993 (Chapter6)
คาร์โบไฮเดรต	51.68	กรัม/100 กรัม	AOAC International,1993 (Chapter1)
โปรตีน	13.62	กรัม/100 กรัม	Based on AOAC (2005) 979.09
ไขมัน	0.32	กรัม/100 กรัม	Based on AOAC(2005) 922.06
เถ้า	24.44	กรัม/100 กรัม	Based on AOAC (2005) 945.46
ความชื้น	9.94	กรัม/100 กรัม	AOAC (2005) 990.20

(นางสาวสุภาวดี วิจารณ์)
ผู้จัดการแผนกเคมี

(นางสาวธารีณี วัฏชนิน)
กรรมการผู้จัดการ

รายงานฉบับนี้รับรองเฉพาะตัวอย่างที่ทดสอบเท่านั้น
ห้ามคัดถ่ายโฆษณาผลการทดสอบและเพียงบางส่วนโดยไม่ได้รับอนุญาตจากห่อปฏิบัติการเป็นลายลักษณ์อักษร

FM-CS-2001 (130705)

256/1 ซอยรัชดาภิเษก ถนนรัชดาภิเษก แขวงจันทรเกษม เขตจตุจักร กรุงเทพฯ 10900 โทร. 0-2938-1131-3, 0-2511-6113-5 โทรสาร 0-2512-3821, 0-2511-6114
256/1 Soi Ratchadapisek Tesaban, Ratchadapisek Road, Chatuchak, Bangkok 10900, Thailand. Tel. 0-2938-1131-3, 0-2511-6113-5 Fax: 0-2512-3821, 0-2511-6114

BIOGRAPHY

Name	Miss Kevalin Vongthoung
Date of Birth	April 20, 1976
Educational Attainment	1998: Bachelor of Science (Medical Technology)
	2004: Master of Science (Tropical Medicine)
Work Position	Medical technologist
	Faculty of Medicine, Ramathibodi Hospital,
	Mahidol University
Scholarship	2010-2014: Mahidol University scholarship

Publications

Jampangern, W., Vongthoung, K., Jittmittraphap, A., Worapongpaiboon, S., Limkittikul, K., Chuansumrit, A., Tarunotai, U., Chongsa-nguan, M. (2007). Characterization of atypical lymphocytes and immunophenotypes of lymphocytes in patients with dengue virus infection. *Asian Pac J Allergy Immunol*, 25(1), 27-36.

Work Experiences	1999-2016: Medical technologist
	Faculty of Medicine, Ramathibodi Hospital,
	Mahidol University