# THE COMBINATORIAL EFFECT OF METFORMIN AND P38-MAPK INHIBITOR ON MYOCARDIAL ISCHEMIA/REPERFUSION INJURY IN DIABETIC RAT

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## ABSTRACT

Type 2 diabetes is a non-communicable disease, which causes morbidity and mortality worldwide. Besides affecting cellular metabolism, type 2 diabetes can lead to various diabetic complications including myocardial ischemia/reperfusion (I/R) injury. Basal p38 Mitogen-activated protein kinase (MAPK) activation has been shown to increase which aggravates cardiac cell death in diabetic hearts. Therefore, inhibition of p38 MAPK activation could be a therapeutic strategy for diabetic cardiomyopathy. Recent study has been shown that metformin, an anti-diabetic drug, exerts cardioprotective effect during I/R condition. Therefore, in this study, we aim to investigate the cardioprotective effect of the combination between metformin and p38 MAPK inhibitor (SB203580) in rat cardiac myoblast cells line (H9c2 cell) under hyperglycaemia and type 2 diabetic model (Goto-Kakizaki (GK) Rat) subjected to myocardial I/R injury. The H9c2 cells were induced to get hyperglycaemic condition using 33 mM of high-glucose solution. The hyperglycaemic cardiac cells were treated with either metformin, SB203580 or the combination between metformin and SB203580 for 24 h follow by I/R induction. The cell viability and cellular reactive oxygen species (ROS) levels were determined using MTT cell survival assay and dichlorofluorescin diacetate assay, respectively. The cellular signalling was determined by Western blotting. The results showed that high-glucose solution did not reduce cell

viability but significantly increased ROS production. In addition, the hyperglycaemic cells subjected to I/R condition significantly decreased cell viability. Treatment with the combination between metformin and SB203580 could reduce ROS level and cell death in hyperglycaemic cells subjected to I/R injury ($p$ <0.05). Molecular studies reveal that combination of both drugs could inhibit p38 MAPK activation and activate Akt phosphorylation.

The beneficial effects of metformin and SB203580 were then determined in in vivo using Wistar rats as a control and GK rats as a Type 2 diabetes rats. After the GK rats were diagnosed to get diabetic condition, they were started to treat with both drugs for 4 weeks. Plasma biochemical parameters and cardiac function were measured before and after drug treatment. Cardioprotective effect against I/R injury was performed by an ex vivo Langendorff perfusion system. The hearts were subjected to 30 min of stabilization, 30 min of ischemia, and 90 min of reperfusion. The infarct size and the signalling transduction were assessed by triphenyl tetrazolium chloride staining and Western blotting techniques, respectively. The results showed that combination treatment with metformin and SB203580 could significantly reduce fasting blood glucose, insulin, and Haemoglobin $A_{1c}$ ($HbA_{1c}$) levels in diabetic condition. Echocardiographic parameters revealed that GK rats demonstrated hypertrophic cardiomyopathy. Treatment with both drugs could reduce myocardial wall thickness of GK rats. Furthermore, treatment with the combination of metformin and SB203580 in GK rats could significantly reduce the percentages of infarct size as well as significantly inhibit p38 MAPK activation ($p$ <0.05) but not sufficient to activate Akt phosphorylation. In conclusion, treatment with combination of metformin and SB203580 provides anti-diabetic parameters by attenuating diabetic parameters as well as exerts cardioprotective effects against I/R injury under hyperglycaemic condition by reduced cell death, inhibited p38 MAPK and lowered infarct size.

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# ABBREVIATIONS

| | | |
|---|---|---|
| ADA | = | The American Diabetes Association |
| ADP | = | Adenosine diphosphate |
| AMP | = | Adenosine monophosphate |
| ANOVA | = | Analysis of variance |
| APS | = | Ammonium persulphate |
| ATP | = | Adenosine triphosphate |
| bpm | = | Beats per minute |
| cAMP | = | Cyclic- Adenosine monophosphate |
| cDNA | = | Complementary DNA |
| DCFH-DA | = | Dichlorodihydrofluorescein |
| DI $H_2O$ | = | De-ionised water |
| DM | = | Diabetes mellitus |
| DMEM | = | Dulbecco's modified Eagle's medium |
| DNA | = | Deoxyribonucleic acid |
| DN | = | Diabetic nephropathy |
| CO | = | Cardiac output |
| DR | = | Diabetic retinopathy |
| EDTA | = | Ethylenediaminetetra-acetic acid |
| EDV | = | End-Diastolic volume |
| EF | = | Ejection fraction |
| ESV | = | End-Systolic volume |
| FAD | = | Flavin adenine dinucleotide |
| FADH2 | = | Reduced Flavin adenine dinucleotide |
| FPG/FBG | = | Fasting plasma glucose/ Fasting blood glucose |
| FFAs | = | Free Fatty Acids |
| FMN | = | Flavin mono dinucleotide |
| FMNH | = | Reduced Flavin mono dinucleotide |
| GDM | = | Gestational diabetes mellitus |
| GLUT4 | = | Glucose transporter 4 |
| GK rat | = | Goto-Kakizaki rat |

## ABBREVIATIONS (CONT.)

| | | |
|---|---|---|
| $HbA_{1c}$ test | = | Glycated haemoglobin $A_{1c}$ test |
| H9c2 | = | Rat cardiac myoblast cell line |
| HR | = | Heart rate |
| IDDM | = | Insulin dependent diabetes mellitus |
| IDF | = | The International Diabetes Federation |
| IL | = | Interleukin |
| i.p. | = | Intra-peritoneal |
| IR | = | Insulin Receptor |
| I/R | = | Ischaemia/Reperfusion |
| IRSs | = | Insulin receptor substrates |
| IVSd | = | Interventricular septum thickness at end-diastole |
| IVSs | = | Interventricular septum thickness at end-systole |
| JNK | = | C-Jun N-terminal kinase |
| Kb | = | Kilobase |
| kDa | = | Kilodalton |
| K-H | = | Krebs-Henseleit |
| LVIDd | = | Left ventricular internal dimension at end-diastole |
| LVIDs | = | Left ventricular internal dimension at end-systole |
| LVPWd | = | Left ventricular posterior wall thickness at end-diastole |
| LVPWs | = | Left ventricular posterior wall thickness at end-systole |
| MAPK | = | Mitogen-activated protein kinase |
| MAPKKK | = | Mitogen-activated protein kinase kinase kinase |
| MAPKAPK2 | = | Mitogen-activated protein kinase-activated protein kinase 2 |
| MEF | = | Monocyte enhance factor |
| MKK | = | Mitogen-activated protein kinase kinase |
| MKP | = | Mitogen-activated protein kinase phosphatase |
| MTT | = | 3-(4,5-dimethylthiaziazol-2-yl)2,5-diphenyl tetrazolium bromide |
| MW | = | Molecular weight |
| NADH | = | Nicotinamide adenine dinucleotide |

## ABBREVIATIONS (CONT.)

| | | |
|---|---|---|
| NADPH | = | Nicotinamide adenine dinucleotide phosphate |
| NIDDM | = | Non-insulin dependent diabetes mellitus |
| nm | = | Nanometre |
| OGTT | = | Oral Glucose Tolerance Test |
| PAGE | = | Polyacrylamide gel electrophoresis |
| PBS | = | Phosphate-buffered saline |
| PI3-kinase | = | Phosphatidylinositol 3-kinase |
| PKB | = | Akt or protein kinase B |
| PKC | = | Protein kinase C |
| ROS | = | Reactive oxygen species |
| RPG | = | Random plasma glucose |
| SDS | = | Sodium dodecyl sulphate |
| SEM | = | Standard error of the mean |
| sI/R | = | Simulated Ischaemia/Reperfusion |
| SV | = | Stock volume |
| TBST | = | TBS-Tween |
| T2DM | = | Type 2 diabetes |
| TEMED | = | NNNN-tetraethylethalinediamine |
| TZD | = | Thiazolidinediones |
| TLR | = | Toll-like receptors |
| TNF | = | Tumour necrosis factor |
| TRIS | = | Tris (hydroxymethyl) methylamine |
| TTC | = | Triphenyl tetrazolium chloride |
| WHO | = | The World Health Organization |

# CHAPTER I

# INTRODUCTION

**Statement of purpose**

Type 2 diabetes mellitus (DM) is a chronic metabolic disorder, caused by pancreatic dysfunction to produce adequate amount of insulin or abnormal in insulin signalling and responses (1). Moreover, DM is a non-communicable disease that causes major global health problems. The International Diabetes Federation (IDF) predicted the DM will be increased to 693 million in 2045 (2), especially type 2 diabetes or insulin resistance (3). In advanced stages of diabetes, there are many type of complications that can be occurred such as retinopathy, neuropathy, nephropathy and cardiomyopathy (4). It has been shown that diabetic patients have two to four folds higher risk of cardiovascular disease than that of non-diabetic patients (5) especially the ischemia heart disease, which is a major cause of mortality in diabetes patients. Type 2 diabetes has been demonstrated to increase the incidence of myocardial injury, infarction and enhance susceptibility to myocardial ischemia/reperfusion, which is caused by the high basal level of p38-MAPK activation in the heart.

p38 Mitogen Activate Protein Kinase (MAPK) is a family of serine/threonine protein kinases that plays an important role in cellular stresses. In particular, p38-MAPK can be activated during insulin resistance, as well as diabetic models and during myocardial ischemia/reperfusion (6). These could aggravate lethal cardiac injury. In addition, the inhibition p38 MAPK activity has been shown to reduce myocardial injury and infarction (6-11). Therefore, the inhibition of p38 MAPK by using pharmacological inhibitors may possibly benefit to reduce myocardial ischemia in diabetes. Moreover, it has been reported that p38 MAPK inhibitors could improve left ventricular functions (12-14), endothelial function (15), and reduced cardiac inflammation in insulin resistance or diabetic models (16). p38 MAPK inhibitor (SB203580) could also improve cardiac contractile functions in insulin resistance (17). These findings point out the usefulness of p38-MAPK inhibition in diabetes.

Metformin is the anti-diabetic drug that commonly used to treat diabetes (18). The previous reports demonstrated that metformin could improve hyperglycaemic

condition or insulin resistance condition (19, 20). Moreover, it has been showed that metformin could improve cardiac function and showed cardioprotective effect in ischemia/reperfusion injury condition (21). Therefore, it postulated that using metformin in diabetic patients or diabetic patients with myocardial ischemia possibly progressive the clinical outcomes. So, combine treatment with metformin and p38 MAPK inhibitor (SB203580) may be provided additional beneficial effects in diabetic heart with myocardial ischemia/reperfusion injury. However, the information about inhibition of p38-MAPK in ischemia/reperfusion injury in diabetes has never been studied.

Therefore, this study will investigate the combinatorial effects of metformin and p38-MAPK inhibitor (SB203580) on the cardiac cells line (H9c2 cell) that induced diabetic subjected to stimulated ischemia/reperfusion injury condition. Then, the effect of combinatorial treatment of metformin and p38 MAPK inhibitor (SB203580) on infarct size, cardiac functions as well as cellular signalling changes, related to diabetic condition complicated with myocardial ischemia/reperfusion condition, also is intensively determined. The understanding regarding the role of metformin and p38-MAPK inhibitor on diabetic heart obtained from this study will certainly provide promising therapeutic as well as preventive strategies at the clinical level in diabetes patients in the future.

**Objectives**

1. To determine the combinatorial effect of between metformin and p38 MAPK inhibitor (SB203580) in cardiac cell subjected to hyperglycaemic condition, as well as, the cellular signalling in response to combination drugs in cardiac cell during hyperglycaemia condition.

2. To determine combinatorial effect of metformin and p38 MAPK inhibitor (SB203580) in cardiac cell subjected to hyperglycaemia following simulated ischemia/reperfusion, together with the cellular signalling in response to combination drugs in cardiac cell during hyperglycaemia and ischemia/reperfusion injury.

3. To determine the combinatorial effect of metformin and p38 MAPK inhibitor (SB203580) in type 2 diabetes animal model, as well as, the cellular signalling in response to combination of the drugs.

4. To determine the combinatorial effect of metformin and p38 MAPK inhibitor in pre-diabetes (insulin resistance) or type 2 diabetes animal model subjected to myocardial ischemia/reperfusion, as well as, the cellular signalling in responses to the combination drugs.

**Hypothesis**

1. The combination between metformin and p38 MAPK inhibitor (SB203580) provide cardioprotective effect in which reducing cardiac cell death during hyperglycaemic condition. In addition, the insight cell signalling of the combination drugs, which reduce cardiac cell death, is investigated.

2. The combination between metformin and p38 MAPK inhibitor (SB203580) provide cardioprotective effect in which reducing cardiac cell death in hyperglycaemic condition and subjected to simulated ischemia. In addition, the insight cell signalling of the combination drugs, which reduce cardiac cell death in this condition, is investigated.

3. The combination between metformin and p38 MAPK inhibitor (SB203580) could reduce plasma glucose, as well as, diabetes parameters in pre-diabetes (insulin resistance) or type 2 diabetes animal model. Moreover, the cellular signalling in response to the combination drugs is investigated.

4. The combination between metformin and p38 MAPK inhibitor (SB203580) shows cardio-protective effects in which reduce myocardial infarct size, improve cardiac function in pre-diabetes (insulin resistance) or type 2 diabetes animal model. Moreover, the cellular signalling in organ level is investigated.

**Scope of the study**

Investigation the *in vitro* effects of combination between metformin and p38 MAPK inhibitor (SB203580) in cell death of cardiac cell (Cardiac-myoblast cell line; H9c2 cell) induced to hyperglycaemic condition in an *in vitro* simulated ischemia/reperfusion injury, as well as the signal transduction in response to the combination drugs in cardiac cell induced to hyperglycaemia following I/R injury. Moreover, this study also examines the combinatory effects of metformin and p38 MAPK inhibitor *in vivo* by using pre-diabetes (insulin resistance) or type 2 diabetes animal model (male Wistar rats and Goto-Kakizaki (GK) Rats) and the effect combined

p38 MAPK inhibitor with metformin in type 2 diabetes induced animal subjected to the *ex vivo* ischemia/reperfusion condition is also determined both of cardiac function and molecular mechanism.

# CHAPTER II

# LITERATURE REVIEWS

## Diabetes mellitus (DM)

### 1. Definition and type of DM

Diabetes mellitus (DM) is a chronic metabolic disorder. It results from dysfunction of pancreas to produce adequate amount of insulin or abnormal insulin signalling and responses consequence to chronic hyperglycaemia (1). Moreover, DM is non-communicable diseases, which causes major global health problem. The World Health Organization (WHO) has predicted the deaths from DM will double between 2005 and 2030 (3). In addition, it has been reported from The International Diabetes Federation (IDF) that diabetes will be increased to 693 million in 2045 (2). Insulin resistance is a prediabetes state, which target cells cannot be activated by insulin hormone to control blood sugar level, although the level of insulin is in a normal range or insulin target tissue do not respond to insulin stimulation (22). The American Diabetes Association (ADA) classified the diabetes into four categories based on the etiology of disease as following

1.1 Insulin dependent diabetes mellitus (IDDM) or type 1 diabetes is a result of β- cell in pancreas cannot produce an adequate amount of insulin for controlling the normal plasma glucose level that resulting in hyperglycaemia condition (23).

1.2 Non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes is insulin resistance, causes by insulin membrane signalling in target organs dysfunction, although the insulin can normally secreted by pancreas (23).

1.3 Other specific types of diabetes or type 1.5 is a result of abnormality or dysfunction in β-cell lead to produce of insufficient insulin hormone, genetic defects of insulin action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug or chemical-induced (23).

1.4 Gestational diabetes mellitus (GDM) is a hyperglycaemic condition occurs during the second or third trimester of pregnancy. It has been reported that about 30 -50 % of GDM patients had possibility progressed to type 2 DM after childbirth (24).

**2. Risk factors**

Type 2 diabetes (T2DM) is a type of diabetes mellitus that could most occurs in the world because it is generated by modified factor and non-modified factor including (25)

2.1 Overweight

The primary risk factor for obesity T2DM is overweight. The high amount of fatty tissue may cause insulin resistant (18). However, sometime being overweight alone does not develop to T2DM.

2.2 Abdominal obesity

The person who has fat distribute primarily at abdomen have higher risk of type 2 diabetes than the person who has deposit in other part of the body.

2.3 Physical inactivity

The less active are greater risk of T2DM than the more active. Besides, many physical activities will help to control body weight, which cause glucose uptake as energy and makes cells more sensitive to insulin.

2.4 Age

The older age, after age 45, is greater risk of T2DM than the younger (23), because the older people tend to do less exercise, loss of muscle mass, and gain more weight. However, T2DM can also development in children, adolescents and younger adults.

2.5 Family history

The risks of T2DM will increases when the parent or sibling has T2DM.

**3. Mechanisms of type 2 diabetes mellitus (T2DM)**

T2DM is a chronic metabolic disorder caused by genetic and environmental factors, which leading to impair in glucose homeostasis. This impairment results in hyperglycaemia condition that involve in the progressive of pathophysiological changes characterized by insulin resistance and chronic inflammation (18).

3.1 Insulin resistance

The insulin signalling is involved in binding of insulin and insulin receptor (IR), which activate tyrosine kinases result in tyrosine phosphorylation of insulin receptor substrates (IRSs). Activation of IRSs can lead to phosphorylation in phosphoinositide 3-kinase (PI3K), then it can activate the downstream such as Akt/protein kinase B (PKB) and protein kinase C (PKC). Afterwards, activated Akt phosphorylates could activate glucose transporter (GLUT4) moves to the cell surface to transport glucose into the cell. In addition, insulin could associate with the RAS–mitogen-activated protein kinase (MAPK) pathway (18, 26).

The insulin resistance (prediabetes) in obesity and T2DM has mainly associated with a worsening of the PI3K pathway. It is known that the cause of insulin resistance is usually related with increased serine phosphorylation of IRS proteins and inhibit or reduce the tyrosine phosphorylation. In some case, the insulin resistance was encouraged by increased IRS degradation in serine phosphorylation.

3.1.1 Phosphoinositide 3-kinase (PI3K)

The PI3-kinase family is divided into four different classes: class I, class II, class III, and class IV. The class I, class Ia, is associated with insulin signalling. The class Ia PI3K is comprise with regulatory subunit (p85) and a catalytic subunit (p110). The normal PI3K pathway is work by between the free p85 monomer and the p85-p110 heterodimer. Moreover, PI3K is require phosphorylation of PI 4,5-biphosphate to PI 3,4,5-trisphoshpate (PIP3) for activating downstream molecules, including serine-threonine kinases, tyrosine kinases, GTPases, and others (26). Protein kinase B (PKB)/Akt is one key downstream target of PI3K, which is activated via serine and threonine phosphorylation (26). Insulin resistance condition is associated with impairment of the PI3K pathway, in which imbalance the free p85 monomer and the p85-p110 heterodimer. The evidence studies in insulin resistant states induced by obesity or type 2 diabetes have been show that increasing the expression of the free p85 subunit could reduce the PI3-kinase insulin signalling, result in insulin resistance condition. Moreover, it has been reported on *in vitro* studies that increasing serine phosphorylation may cause by inhibition or reducing of PI3K activation (27).

3.1.2 Protein Kinase B or Akt (PKB or Akt)

Akt is the serine/threonine kinase Akt, a downstream mediator of PI3K. It has been divided into 3 isoforms, Akt1, Akt2, and Akt3, all of which are extensively expressed in the tissues. Akt plays an important role by linking glucose transporter (GLUT4) to the insulin signalling pathway. In type 2 diabetes model, Akt2 could cause of impairment in insulin activity by incapable to phosphorylate downstream targets and to mediate inhibition of phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenic key enzyme (26-28), resulting in insulin resistance.

In addition, the causes of increased serine phosphorylation are multifactorial, including ectopic lipid accumulation, mitochondrial dysfunction and inflammation (18).

3.2 Ectopic lipids and PKCs.

The obesity and lipid accumulation in ectopic tissues, skeletal muscle and liver, such as fatty acyl CoAs and diacylglycerol can be attributed to metabolic diseases including insulin resistance and type 2 diabetes (29). The lipid accumulation can induce insulin resistance by increasing tissue diacylglycerol (DAG) levels (30), which lead to activation of members of PKC isoforms, PKCθ in muscle (31), and PKCδ (18) and PKCε (18) in the liver. Then, these PKCs can phosphorylate serine residues in IRS proteins result in impairment of insulin signalling.

3.3 Mitochondrial dysfunction.

The mitochondrial dysfunction can contribute to insulin resistance in multiple ways. It has been investigated in many organs such as liver, muscle, adipose tissue and brain of several obesity and T2DM model, that suggested increasing levels of reactive oxygen species, which activate redox-sensitive serine kinases to phosphorylate IRS proteins and produce insulin resistance, but this finding remains unclear (18).

3.4 Inflammation.

The mechanistic links between obesity-induced inflammation and insulin resistant states can elucidate by the accumulation of free fatty acids (FFAs). Obesity could activate pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin (IL-8) responses in vascular endothelial cells and adipocytes. The increasing level of these pro-inflammatory cytokines can induce insulin resistance by activating downstream kinases, including IκB kinase-β

(IKKβ), JUN amino-terminal kinase 1 (JNK1; also known as MAPK8) and p38 MAPK, which can contribute to the phosphorylation of serine residues in IRS proteins (32) and stimulate production of suppressors of cytokine signalling (SOCS), which inhibit of the action of IRS proteins (33) result in insulin resistance. Moreover, increased FFA levels can also increase the activity of IKK and JNK, which leading to insulin resistance by serine phosphorylation of IRS proteins and block IRS tyrosine phosphorylation (34).

The previous studies, which above mentioned, have evidences suggested that the obesity, lipid accumulation, increasing FFA level, mitochondrial dysfunction, increasing ROS level, and inflammation are a risk factor of impairment in insulin signalling. This impairment leads to insulin resistance, which is a worsening of the PI3K pathway and reduce activity of IRS protein, finally progressive to type 2 diabetes mellitus. (Figure1)

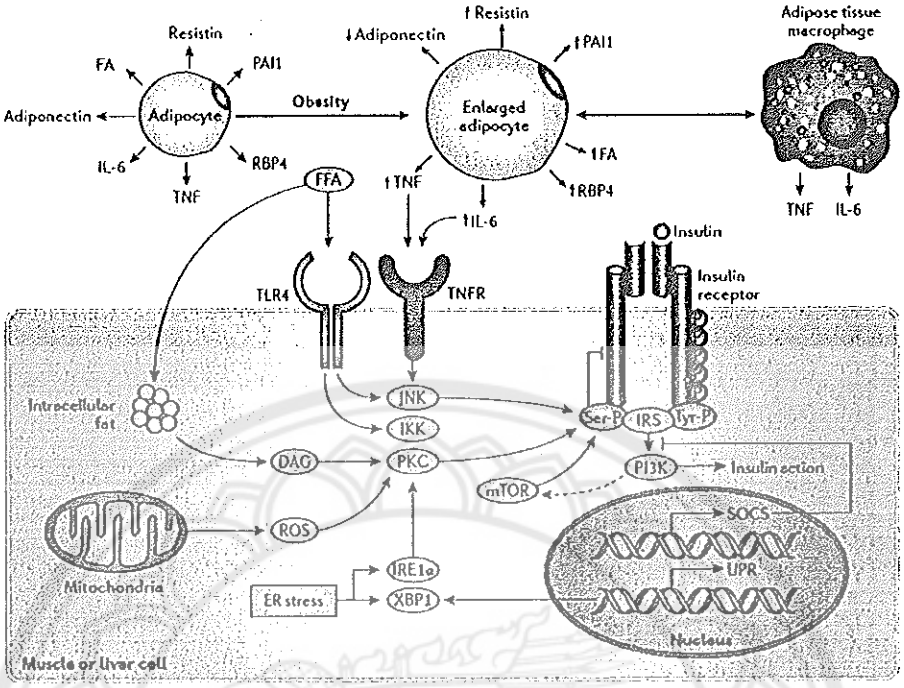


Figure 1 Mechanisms of insulin resistance. The obesity (accumulated free fatty acids (FFAs)), inflammation response (pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor (TNF) and resistin) and mitochondrial dysfunction (cause of released reactive oxygen species (ROS)) are a risk factor of impairment of insulin signalling via activation of diacylglycerol (DAG), (JUN amino-terminal kinase (JNK), IκB kinase (IKK) and nuclear protein kinase C (PKC), respectively. These factors can lead to increase insulin receptor substrate (IRS) serine phosphorylation, result in insulin resistance. Insulin resistance can reduce activation of phosphatidylinositol 3-kinase (PI3K) pathway as a result of development to type 2 diabetes (18).

**4. Diagnosis of diabetes**

The criteria for diagnosis of diabetes divided into 3 categories including high level of fasting plasma glucose (FPG) or high random plasma glucose (RPG), the impairment of oral glucose tolerance test (OGTT), the high amount of glycated haemoglobin test ($HbA_{1c}$ test) (24). The criteria for diagnosing diabetes are showed in Table 1. In fasting plasma glucose (FPG), the FPG level at 80-100 mg/dL, over 100 mg/dL and over 126 mg/dL is concerned as normal, patients at a risk of diabetes and diagnosed diabetes, respectively. The normal level of RPG is between 70- 120 mg/dL, whereas, the random plasma glucose level at 121-199 mg/dL is considered as risk of diabetes. The RPG higher than 200 mg/dl will be considered as DM. The normal OGTT level is lower than 140 mg/dL. In the patients with high risk of diabetes and diabetes show the OGTT between 141 – 199 mg/dL and greater than 200 mg/dL, respectively. Moreover, the normal level of $HbA_{1c}$ is below 5.7% and 5.8-6.4 % in normal and patients at risk of diabetes, whereas, in diabetes patients is greater than 6.5% (18). (Table 1)

**Table 1 Criteria for diagnosis of diabetes**

| Test | Normal range | Patients at risk | DM patients |
|---|---|---|---|
| FPG | 80-100 mg/dL | 101 - 125 mg/dL | ≥ 126 mg/dL |
| RPG | 70-120 mg/dL | 121- 199 mg/dL | ≥ 200 mg/dL |
| OGTT | < 140 mg/dL | 141 - 199 mg/dL | ≥ 200 mg/dL |
| $HbA_{1c}$ | < 5.7 % | 5.8 - 6.4 % | ≥ 6.5 % |

**Note:** Abbreviations: FPG; fasting plasma glucose, RPG; random plasma glucose, OGTT; oral glucose tolerance test, $HbA_{1c}$; glycated haemoglobin test

**Source:** modified from American Diabetes (18, 24).

**5. Symptoms of diabetes**

In general, signs and symptoms of DM includes polydipsia, polyurea, weight loss, cramps, polyphagia, candidiasis, blurred vision, constipation and fatigue (23, 35). These signs and symptoms are commonly found in both of type 1 and type 2 diabetes. However, some patients has been specific clinical complications and severe complications (36).

**6. Complications of diabetes**

Prolonged hyperglycaemic condition or the progression of diabetes results in development of diabetic complications. The complications in diabetes were divided into 2 major types. First, micro-vascular complication including retinopathy, nephropathy and neuropathy (4). The second type is macro-vascular complication such as coronary heart disease and peripheral vascular disease (4, 37).

6.1 Diabetic retinopathy

Diabetic retinopathy (DR), the pathology of the eye found in diabetes, is a visual loss or potentially blinding, as a result of microvascular damages, micro-aneurysms (38). The lipoproteins and blood are leaked from tiny blood vessels that nourishing the retina. After that, the macular oedema is occurred in the eye, nerve-fibre layer infarction, intra-retinal microvascular abnormalities (collateralization), and the venules of the eye are dilated. Finally, the optic disk and retina (vessels) is irregular, proliferation of fibroblasts and vitreous haemorrhage (the proliferative changes) is also occurred (37, 38). It has been reported in the patients with IDDM have high risk with DR as early as 5 years, while NIDDM 20 years after diagnosis of diabetes (39).

6.2 Diabetic nephropathy

Diabetic nephropathy (DN) is a condition of dysfunction in the glomerular basement membrane becomes more thickness, hypertrophy of the kidney, nodular and diffuse glomerulosclerosis, tubular atrophy, and interstitial fibrosis (40). These symptoms are concerned as structural abnormalities (40) and functional kidney

abnormalities as a result of proteinuria due to increase in glomerular filtration rate, systemic hypertension, and loss of renal function or renal failure (41). The IDDM patients have risk of final stage DN around 50% - 75% (40). After an onset of diabetes, the progression to DN in type 2 DM patients is dramatically higher than type 1 DM patients (40).

6.3 Diabetic neuropathy

Diabetic neuropathy is a microvascular complication of diabetes that is associated with neurological abnormalities. In pre-diabetes, the neurological abnormality in the distal lower extremities was reported (42). The complications of neuropathy in diabetes are not only morbidity in distal symmetrical neuropathy or polyneuropathy but also involve several pathophysiology, such as the severe pain, loss of ambulation and increased risk of foot ulceration and amputation (43).

6.3.1 Ulceration

Ulceration is a general complication in diabetic patients that a condition of limb pathology, including ulcerations, infections, and gangrene, cause by peripheral arterial disease (PAD) (44, 45). The imbalance of blood demand and supply to limb regions can finally cause peripheral ischemia, infarction, diabetes induced peripheral neuropathy (DPN), and foot deformation (46). It has been reported that the incidence of diabetic foot ulcers in diabetes patients is up to a 25% (46).

6.4 Diabetic cardiomyopathy

Diabetic cardiomyopathy is a condition of dysfunction in ventricular, which is occurred independently of coronary artery disease and hypertension (47). Moreover, characteristic of diabetic cardiomyopathy may be included diastolic dysfunction, structural abnormalities of left ventricle, left ventricular hypertrophy (LVH), systolic and diastolic impairment and myocardial ischemia (48-50), which will be described as following.

6.4.1 Coronary heart diseases

The coronary heart diseases or cardiovascular diseases are a major complication and critical complication in diabetic patients that cause of early death in diabetes about 65 % (5). The patients who have chronic hyperglycaemia have a higher risk of cardiovascular diseases than non-diabetic subject around two to four times (5). This highlight the significant of cardiovascular complication in all types of diabetes (51).

6.4.2 Left ventricular hypertrophy (LVH)

Left ventricular hypertrophy (LVH) is referred to an increase in LV mass, which is a risk factor for heart failure, due to uncontrollable hypertension contribute to reduced myocardial compliance, finally cause of heart failure (52). The Strong Heart Study conducted in Native Americans reported the LV mass and wall thickness could occur higher in both men and women with diabetes (52). In addition, previous studies found the correlation of diabetes patients was an increased in LV mass, LV thickening, and increased risk of heart failure (53, 54).

6.4.3 Systolic dysfunction (SD)

Systolic dysfunction, which usually happens after diagnose is of diastolic dysfunction (DD), is a condition of loss of contractile activity lead to reduction in the ejection fraction (EF) and enlargement of the LV chamber (55). Previous studies in an *in vivo* animal model have been demonstrated that both IDDM and NIDDM delayed LV relaxation cause increased in LV thickening (56, 57). Diabetic subject who is diagnosed SD have higher risk of mortality than the diabetic subjects without SD (58).

6.4.4 Diastolic dysfunction (DD)

Diastolic dysfunction is the relaxation activity of the heart dysfunction owing to reduce in end diastolic volume, which lead to poor stroke volume (SV) and cardiac output (CO) (52). It has been reported that the cause of contractile and diastolic dysfunction was the accumulated of triglyceride and changed in $Ca^{2+}$ level in the heart of type 2 diabetes subjects (59). This condition result in the sarcoplasmic reticulum (SR) - $Ca^{2+}$ leak in diastolic period and the inactivation of $Ca^{2+}$ induced $Ca^{2+}$

release (59). In addition, the accumulation of intracellular $Ca^{2+}$ not only cause diastolic dysfunction but also induce the injury and inflammation of cardiac myocytes (59). The healing process that activated after injury was loss of healing regulation resulted in cardiac fibrosis, which is also a cause of systolic and diastolic dysfunction (60, 61).

6.4.5 Heart failure (HF)

The heart failure in diabetes is a condition of the insufficient oxygen and blood supply to the body result from the reduction of heart's pumping activity. It have been reported that the incident of heart failure in diabetes patients were around 20-25% (62). In addition, it has been reported about the incidence of congestive heart failure in type 2 diabetes that diabetic patients have higher risk develop to heart failure than non-diabetic patients about 2.5 fold (63) .

6.4.6 Ischemic heart disease (IHD)

Ischemic heart disease refers to lack of blood supply to heart tissue lead to area of tissue death. Diabetic patients have two to four folds higher risk of ischemic heart disease than the subjects without diabetes (5). Moreover, Ischemic heart disease has been identified as an important factor of death and illness in diabetes (51). The atherosclerosis is a major cause of ischemic heart disease and lead to coronary artery stenosis. Then the imbalance of blood demand and blood supply in the heart, reduction of coronary flow are consequences to coronary artery stenosis finally, results in ischemia and infarction (64). An inadequate of oxygen and glucose delivery into cardiomyocytes leads to oxidative stress production, cardiomyocytes acidosis, loss of adenosine triphosphate (ATP), mitochondrial dysfunction, and cell death (65, 66). It is well known that the process of electron transport chain in the cellular respiration is require the oxygen for accept electron as an acceptor (67). Moreover, the shifting of energy production in cardiomyocytes from aerobic metabolism to anaerobic metabolism as a result of the loss of oxygen supply to the mitochondria. This events result in accumulation of intracellular lactic acid, proton ($H^+$), and ATP depletion (68). The $H^+$ overloaded, activate Na-H exchanger in plasma membrane, reduced intracellular $H^+$ and increased intracellular $Na^+$, finally contribute to accumulation of $Ca^{2+}$ in cardiomyocytes (69). The amount of calcium that is increased and cause mitochondria swelling, promote

the opening of mitochondria permeability transition pore (MPTP) and the cytochrome C releases in cytosol subsequence to an opening of the MPTP (70). After that, the released cytochrome C can cause of cardiac cell death by stimulating the caspase 3 pathway in which plays a role in apoptotic pathway (71). In addition, the oxidative stress was closely associated with ischemic heart disease by the overload of ROS, which can activate the MPTP and result in cellular apoptosis (72, 73). Moreover, it has been reported that the membrane rupture due to lack of ATP also result in necrotic cardiac cell death (74).

From this observation demonstrates that chronic hyperglycaemia condition or diabetes leads to a worse condition in multi system complications, in particular cardiovascular system that result in mortality and morbidity. Therefore, the treatment of diabetes by control of glycaemic status, prevent diabetic progression and complication will be reduced mortality rate in diabetic patients. The anti-diabetic drugs and the insulin therapy have been used to control of diabetes for several decades. The mechanisms of the treatment of diabetes by anti-diabetes drugs will be discussed later in this study.

**Ischemia/reperfusion injury**

The ischemia heart disease (IHD), which is one of the coronary heart diseases, is a condition of tissue reduces or cut off blood supply to the heart consequence to decrease $O_2$ supply in the heart tissue. An extension of myocardial ischemia leads to decrease of $O_2$ and nutrition, especially glucose, resulting in cellular necrosis and apoptosis. Therefore, the condition of insufficient blood supply in this tissue leads to several pathophysiology in the heart including acute myocardial infarction, cardiac cell death (75), peripheral vascular insufficiency, stroke, and hypovolemic shock (76).

**1. The cause of Ischemic heart disease**

The atherosclerosis is a major cause of ischemia heart disease or coronary heart disease, which is a coronary artery wall thickens or a coronary artery obstructed by fibrofatty plaque (77-80). The accumulation of fatty molecules leads to lack of blood supply in coronary artery of the heart. Finally, the heart muscles are injured (80).

1.1 Atherosclerosis

Atherosclerosis is the most frequent underlying cause of coronary artery disease, carotid artery disease, and peripheral arterial disease (81). The pathology of atherosclerosis is the interruption or blockage in the wall of large arteries by the accumulated fatty molecules and fibrous elements (79). In particularly, the accumulations of low-density lipoprotein (LDL) in the blood vessel, which could induce inflammatory response. The LDL particles can diffuse passively via endothelium cell junction, especially the cells in regions of branching or curvature (77, 79, 82). The native LDL is not encompassed by macrophage that can pass to the wall of the artery and the LDL that retains in the intima is modified by reactive oxygen species (79) by some enzymes such as myeloperoxidase and lipooxygenase, which produce oxidized LDL (oxLDL). This oxLDL could stimulate inflammation. The accumulation of oxLDL was taken in cell by macrophage via scavenging receptors, result in generation of foam cell (77, 79, 82-84). After that, the foam cells or fatty streak was accumulated that are called the first step of atherosclerosis. The platelets aggregation and thrombus formation due to appear of the tissue factors (the primary factor activates clotting formation), leading to an increasing in the plaque size and obstruction of artery blood flow supplying to tissue (79). Finally, the thrombus occurred leading to ischemia of the myocardial muscle by blocked or reduced blood flow to downstream tissue. (Figure 2)

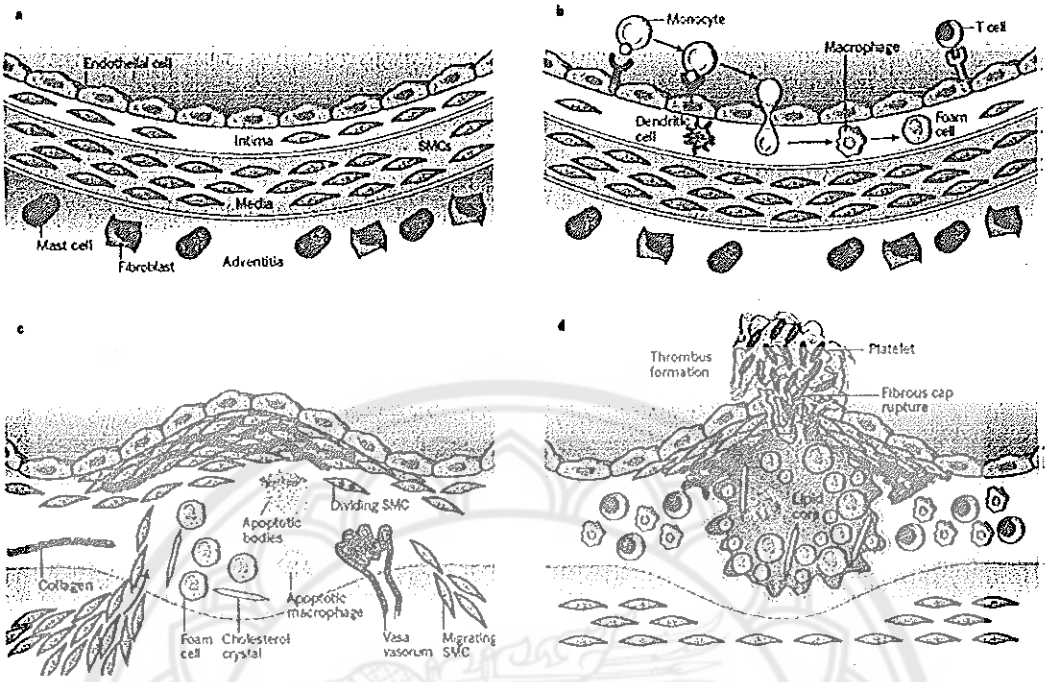


Figure 2 The development of atherosclerotic lesions. a). The normal artery consists of three layers, the inner layer, the tunica intima and the outer layer of arteries. b). The first steps of atherosclerosis are the endothelial was activated by adhesion of blood leukocytes, leading to the bound leukocytes move to the intima. c). The result of the migration of SMCs from the media move to the intima, the development of resident intimal SMCs and media-derived SMCs, and the increased synthesis of extracellular matrix macromolecules including collagen, elastin and proteoglycans. d). Thrombosis, the most complication in atherosclerosis, the most complicates a physical destruction of the atherosclerotic plaque. The thrombus that spread to the vessel lumen can obstruct or reduce blood flow to downstream tissue (78).

**2. Reperfusion injury**

The reperfusion, which is the therapeutic of ischemia, is a condition of renewal blood flow to tissue that occur ischemia condition for prevent tissue necrosis and regain organ function (85). However, reperfusion can also cause of cellular injury, cellular necrosis and apoptosis by increasing of oxidative stress especially oxygen-free radical and $Ca^{2+}$ overload (85) that is explained as Ischemia/Reperfusion injury (I /R injury) (86, 87).

Oxidative stress and reperfusion injury

The Reactive oxygen species (ROS) that was generated by many mechanisms during reperfused myocardium (85). The ROS, especially HO•, can interact with biological macromolecules such as proteins, lipids, and nucleic acids (88). For, in lipid peroxidation, the mechanism of ROS was oxidizing the membrane of the cell and organelles which consist of polyunsaturated fatty acids (PUFA) result in cell membrane damage (88). The plasma membrane of the cell and organelles was ruptured by the lipid peroxidation process, which is a chain reaction of generates the fatty radical, leading to cellular necrosis (88). Besides, the activity of the cell membrane channel such as $Ca^{2+}$ ATPase pump and $Na^{+}$-$K^{+}$ ATPase was affected by the peroxidation of the plasma membrane in the cardiomyocytes (89). For example, the $Ca^{2+}$ can moving through the $Ca^{2+}$ pump, and the accumulation of intracellular $Ca^{2+}$ by the ROS. Finally, the heart is hypercontracture (85). In addition, the DNA and mitochondria are disrupted by the ROS cause to activate cellular apoptosis in intrinsic and extrinsic pathway (89). The mitochondrial membrane potential was destroyed by several proteins including BCL-2-associated X protein (BAX) and BCL-2 antagonist or killer (BAK), which was activated by the B cell lymphoma 2 (BCL-2) homology 3 (BH3)-only proteins, cause to release many apoptotic factors especially, cytochrome C, result in cellular apoptosis (89).

**3. Ischemia/reperfusion injury (I /R injury)**

Ischemia/reperfusion injury is a condition of recovery of blood flow ($O_2$ and nutrition) to the myocardial after a period of insufficient blood supply (ischemia). Ischemia–reperfusion is relevant to the aortic cross-clamping, coronary angioplasty,

thrombolytic therapy, organ transplantation, or cardiopulmonary bypass leading to this local tissue and systemic inflammation. The inflammatory response after I/R may be damage myocardial cell by released pro-inflammatory cytokine (Interleukin (IL)-1, tumour necrosis factor (TNF)-alpha and interferon (IFN)-gamma) and reactive oxygen species (ROS) (90). From this leads to cellular necrosis and apoptosis. Therefore, during myocardial ischemia/reperfusion, the cardiomyocyte will be died by necro-apoptosis (75).

**Anti-diabetes drugs (oral hypoglycaemic drugs)**

Anti-diabetic drugs have been widely used for controlling blood glucose and/or increasing the insulin sensitivity in diabetic patients (91). Controlling blood glucose level results in the improvement quality of life (92). Anti-diabetic drugs are categorized into 4 groups including thiazolidinediones, sulfonylureas, biguanide, and incretins.

**1. Thiazolidinediones (TZD)**

TZD was introduced to the pharmaceutical market and begun to use for treating in 1997 (91). TZD has several types of anti-diabetic drugs such as pioglitazone, rosiglitazone, ciglitazone, and troglitazone. The main mechanistic action of these drugs is their ability to stimulate nuclear peroxisome proliferators-activated receptor (PPAR)-$\gamma$. The consequences of stimulated PPAR-$\gamma$ receptor involve the expression of gene that plays a role in enhancing insulin sensitivity, (93) and genes that involved in carbohydrate and lipid metabolism (94). Moreover, activated PPAR-$\gamma$ can also induce the glucose uptake into the cell (21). Thus, glucose level was decreased by improving insulin activity and the ability to enhance glucose uptake.

**2. Sulfonylureas**

Sulfonylureas have a several members such as Glibenclamide, gliclazide, glipizide, and glimepiride. The activity of sulfonylureas to reduce blood glucose has been published since 1942 (91). It was used in treatment of NIDDM in the past, but was withdrawn from market since the adverse effect on bone marrow was reported (91). The mechanism of action is stimulating the $\beta$-cell in pancreas to secrete insulin into the blood

to reduce blood glucose (95). Sulfonylureas is a sulfonylurea receptor (SRR-1) blocker that can inhibit the opening of ATP potassium channel in β-cell, reducing resting membrane potential that leads to depolarization, opening $Ca^{2+}$ gated channel, thus increasing $Ca^{2+}$ influx and the pre-formed insulin is secreted from β-cell by exocytosis (21).

**3. Incretins**

The incretin is a gastrointestinal peptide hormone, which induces the secretion of insulin and inhibits the secretion of glucagon after food intake (84). The incretin hormone has been divided into glucose dependent insulinotropic peptide (GIP) and glucagon like-peptide (GLP-1) (21). Glucagon like-peptide (GLP-1) is an important hormone, which stimulates the β-cell in pancreas to enhance the insulin secretion (96). However, GLP-1 can be rapidly degraded by dipeptidyl peptidase-4 enzyme (DDP-4) (96). The mechanism of dipeptidyl peptidase 4 (DDP-4) enzymes is to enhance GLP-1 degradation. GLP-1 is an anti-hyperglycaemia hormone, which is secreted in response to food intake, and can stimulate insulin secretion. GLP-1 has very short haft-life in circulation, about 2-5 minute (97). Degradation of GLP-1 by DDP-4 leads to decrease insulin secretion from β-cell resulting in high blood sugar level.

The anti-diabetic drugs in this group have been categorized into 2 groups including GLP-1 analogues such as exendin-4 and liraglutide, and DDP-4 inhibitor such as vildagliptin and sitagliptin. The mechanism of incretin can explain by DDP-4 inhibitor, which inhibits the action of DDP-4 enzyme by forming covalent bond with DDP-4 enzyme and indirectly inhibits GLP-1 degradation and maintain GLP-1 in small intestine and circulation, leading to stimulation of β-cell and subsequently result in increased insulin secretion (21).

**4. Biguanide (metformin)**

Metformin is one of diabetes drugs, which is widely used for treatment for diabetes patient around the world and belong to biguanide drug family (18). Metformin could maintain glucose level in plasma by stabilizing and suppressing hepatic glucose production (91). Moreover, it increases the sensitivity between insulin hormone and heir

receptor resulting in reducing of fasting plasma glucose and $HbA_{1c}$ level (91). In addition, the metformin is a cause of weight loss in diabetes patient. However, it has no effect on β-cell function (18, 91).

Although metformin provide a therapeutic potential in patients who have insulin resistance and diabetes, it can also provide some adverse effects in some patients including treatment failure, heart toxicity (98). As a result of its adverse effect, metformin could increase the mobility and mortality of diabetes patients (98, 99).

4.1 Structure/Mechanisms

Metformin (Biguanide), an anti-diabetes, has been used in patients with overweigh NIDDM since 1957 in the U.K then was approved by U.S. and it was recommended by the American Diabetes Association, using in combination with lifestyle modification in type 2 diabetes (20). It has well been known about critical adverse effect of biguanide concerning "fatal lactic acidosis" leading to low pH in arterial blood that result in multiple organs failure and cause of death in NIDDM subjects (100), but this serious condition has been rarely seen in metformin treatment with appropriate dose (101).

The structure of metformin is a N,N-dimethyl biguanide complex, which as antimicrobial, analgesic, antimalarial or glucose reducing agent as well as antimetabolite for organisms that block the folic acid metabolism (102). The important mechanism of metformin is action in hepatocyte, where gluconeogenesis occurs. Metformin can pass through the cell by binding with its receptor such as SLC22A1, which express on the membrane of hepatocytes. At the cytoplasm, it localizes to the mitochondria and inhibits the complex I of the mitochondria leading to reduce ATP production, result in AMP accumulation. AMP accumulated in the cell can inhibit the adenylate cyclase enzyme by binding with the P-site of this enzyme. The inhibition of this enzyme leads to decrease generation of cAMP, which produce upon stimulation of the glucagon receptor. As a result, the PKA signalling pathway are inhibited, leading to suppress the gluconeogenesis as well as reduce gene expression of glycolytic pathway for example CREB1/PGC-1α. Furthermore, metformin can activate AMPK because of AMP increasing, which suppress fat metabolism and contributes to the decreased gluconeogenic gene expression as well. Moreover, AMPK can also improve insulin

receptor function and improve glucose transport leading to improve insulin sensitivity (103-108) (Figure 3).

In addition, it has been reported that metformin shows a cardio-protective effect by p38 MAPK activation. However, it has been only reported that metformin could cellular apoptosis by p38 MAPK activation. The effects of metformin on p38 MAPK activation in the heart was shown in table 2 (21).

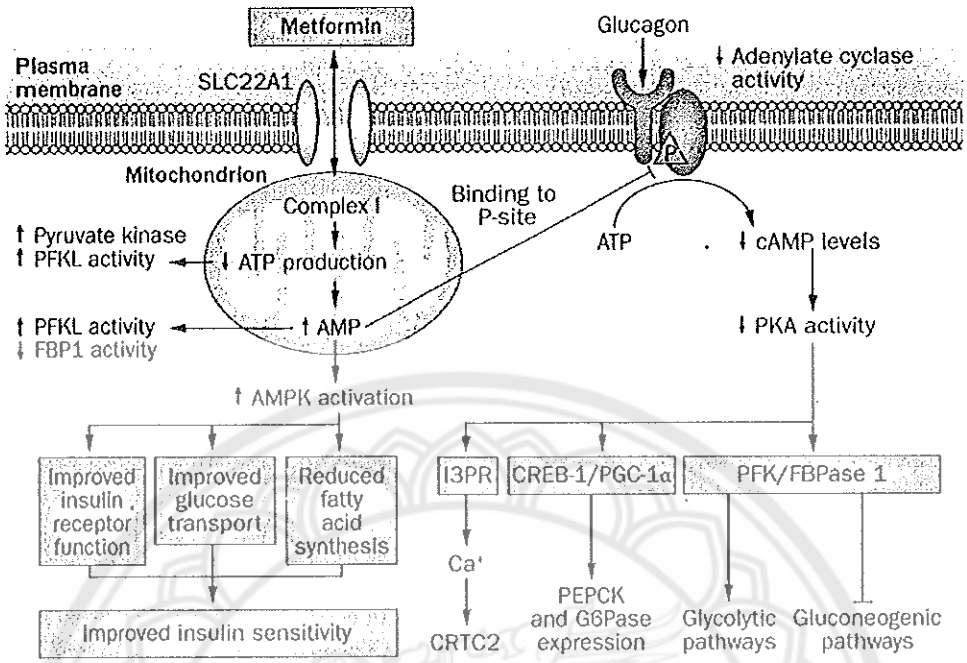


**Figure 3 Model of metformin action in the hepatocyte. Metformin can pass through the cell by binding with its SLC22A1 receptor, and then it can inhibit the complex I of the mitochondria leading to reduce ATP production, result in AMP accumulation. AMP accumulated in the cell can inhibit the adenylate cyclase enzyme, leading to suppress the gluconeogenesis Moreover, metformin can activate AMPK leading to improve insulin receptor function and improve glucose transport result in improve insulin sensitivity (108).**

**Table 2 Effect of Biguanides drugs on myocardial p38-MAPK activation**

| Type of effects | Dosage | Study model | Outcome | Effect on p38-MAPK | References |
|---|---|---|---|---|---|
| Cardio-protective effect | 1 mM Metformin 12 hours | *In vitro*: Adult mice cardiac fibroblast treated with AICAR or Metformin | Metformin dose-dependently increased IL-6 production | ↑p38-MAPK | (109) |
| | 2 mM Metformin | *In vitro*: Rat cardiac myoblast cell line (H9c2) treatment with metformin in buffer containing insulin for 8 hours | *In vitro*: Metformin accelerate glycolysis | *In vitro*: ↑p38-MAPK | (110) |
| | | *Ex vivo*: Isolated male Sprague-Dawley heart perfused with metformin | *Ex vivo*: Metformin enhanced cardiac output, heart rate, and hydraulic work | *Ex vivo*: ↑p38-MAPK | |
| Adverse effect | 3 mM Metformin 30 minutes prior to ischemia | *In vitro*: Neonatal rat ventricular myocytes subjected to 3hrs simulate ischemia | Metformin stimulate Bax translocation in response to p38-MAPK | ↑p38-MAPK | (111) |

**Source:** modified from Sarawut Kumphune, et al. (21)

**MAP Kinase**

**1. Introduction of MAP Kinase cell signalling**

In the molecular level, a series of extracellular signals, cytokines and growth factors can control the cell growth, differentiation, apoptosis, metabolism and other cellular functions. These signals are capable of transmitting into the cells. If the signalling complexes are assembled for appropriate integration and processing, it will lead to a stimulus-specific responses of this signal (112). Protein cascades of protein kinase family have been known as play important roles in signal transduction in eukaryotic cells. (113, 114).

The MAPK pathway is very importance and conserved in all eukaryotic cells (114). They are the cascade of protein kinases, which play a central role in signal transduction responded to many stimuli such as cell stress, cell death, cell proliferation. These cascades consist of at least three protein kinases in series (114). The MAPK cascade is comprised a MAPK and two upstream components including MAPK Kinase (MAPKK or MKK), and MAPKK Kinase (MAPKKK) (112). The important constituent in this pathway are a family of protein-serine/threonine kinases, MAP kinases (for *m*itogen-*a*ctivated *p*rotein kinases), which are activated in response to a variety of signalling molecules such as growth factors and cell stress as well (115). Nowadays, MAPKs are divided into 4 distinct groups in mammals including p38 MAPKs pathways, extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs)/ stress-activated protein kinases (SAPK), and ERK5/BMK1 (Figure 4) (112).

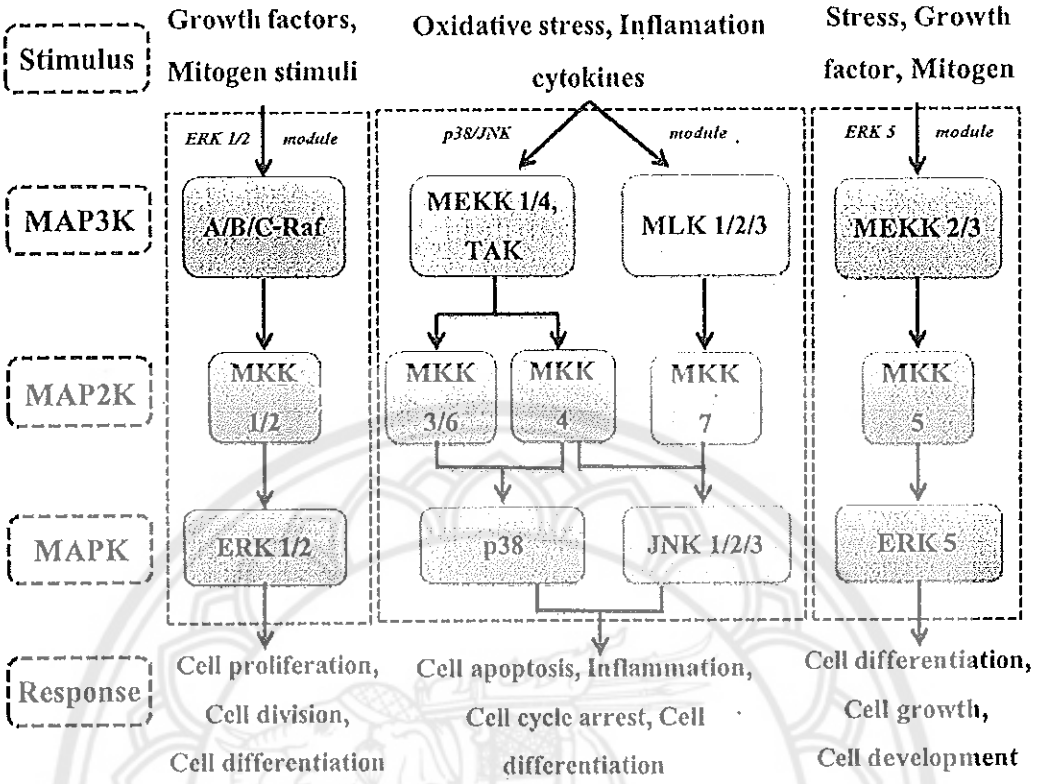


Figure 4 Mitogen activated protein kinase signalling cascades. The MAPK cascade comprises three protein kinases a MAPK and two upstream components, MAPK kinase (MAPKK or MEK) and MAPKK kinase (MAPKKK). It is activated by several factor such as growth factor, Oxidative stress and inflammation cytokine. (116)

**2. MAP Kinase regulation**

MAP Kinases require a transferring of phosphate group from the donors (upstream kinases) for activation called a phosphorylation cascade. Nowadays, the MAP kinase cascades have known as the MAP/ERK kinase (MEK or MKK) families are the upstream kinase of MAPK. They are specificity enzymes that dual phosphorylate the hydroxyl side chains of serine/threonine and tyrosine residues in their MAP kinase substrates (114). The MAP kinases can be classified by Thr-Xaa-Tyr (TXY) that is a sequence of the dual phosphorylation site, in a regulatory loop between kinase subdomains VII and VIII (117). All isoforms of p38-MAP kinase have dual phosphorylation motif (Thr-Gly-Tyr (TGY)), otherwise the ERK1/2 and ERK5/BMK1 phosphorylate at the Thr-Glu-Tyr (TEY) motif and JNK/SAPK group possess the Thr-Pro-Tyr (TPY) motif. The Xaa residue, which located in the dual phosphorylation motif, as well as the loop length, influences substrate specificity (118).

It probably true that the MEK signal can activate the signal transducer from MEK-MAP kinase to increase the collaboration between the activation of the MAP kinase and allow modulation by other signalling events to be amplified. It has been obviously reported that in case of ERK1/2, threonine is phosphorylated after the kinases are phosphorylated on tyrosine (119, 120). Before the phosphorylation of threonine, the tyrosine-phosphorylated protein was accumulated but they are not active. This non-procedure of phosphorylation lead to the instalment of a threshold (119, 120). Then, the kinases are quickly shifted to the active state as threonine is phosphorylated, after this accumulation threshold was reached appropriate level (121, 122).

In addition, the phosphorylation of two residues; either serine or threonine, in their activation loops can activate MEKs (123). At least in the case of MEK1, either phosphorylation alone significantly increases activity, in contrast to the effects of the phosphorylation on the MAP kinase (124).

**3. p38-MAPK**

The p38 MAP Kinase is one of serine/threonine protein kinases that play an important role in cellular responses to external stress signalling and several cellular processes such as cell differentiation, inflammation, cell proliferation and cells death

(125, 126). The p38 MAPK in human was originally isolated as a 38-kDa protein rapidly tyrosine phosphorylated in response to lipopolysaccharide (LPS)-stimulation in human monocytes (126).

The p38 MAPK was classified into 4 isoforms including α, β, γ and δ. The p38α or MAPK 14 is the best characterized and likely be the most physiologically relevant kinase associated with inflammatory responses (127). It has been reported from the gene databases provide useful information that the p38β or MAPK 11 has more than 70% identity to p38α (127, 128). Both α and β isoforms are abundantly expressed in many tissues, but they have different functions (127, 128). Another isoform of p38-MAPK, that classified by the genetic information, are the p38γ (also known as SAPK3 and MAPK12) and p38δ (also known as SAPK4 or MAPK13) (127-131). Both γ and δ isoforms are largely expressed in difference organs, the p38γ isoform is expressed in skeletal muscle, but p38δ is expressed more commonly in many adult tissues and during development (129-131). In comparison of the each p38 isoform sequence shown it has more than 69% identity within this group, but the other MAP kinase family members have only 40 to 45% (118). Therefore, despite their high overall degree of homology and the same preference for phosphorylating serine and/or threonine that precede prolines, at least *in vitro*, the various subfamily members have different specificities for substrates (132).

3.1 p38 MAPK activations and mechanisms

The theory pathway of activate p38 MAPK is involves MAP2K-catalysed that phosphorylate at the threonine and tyrosine residues in the activation loop, leads to conformational changes and result in increasing of the binding to substrates and catalytic activity of p38-MAPKs (133). Indeed, it has been demonstrated in mice gene-targeting experiments that MKK3 and MKK6 play major roles in p38α activation (133). However, the mechanism of stimulation in p38α or p38β was involved in several activators that has been explained in figure 5. The T-cell receptor (TCR), which stimulated by T-lymphocytes, is involve in p38α activation by the TCR-proximal tyrosine kinases that phosphorylated on $Tyr^{323}$. From this, leads to p38α autophosphorylation on the activation loop, result in increasing the activity of kinase into substrates (134). Growth-arrest and DNA-damage-inducible protein or GADD that

act as an endogenous inhibitor in another pathway of p38α-activation in T-cells, is bind to p38α and protect phosphorylate at $Tyr^{323}$ (135). It have been reported about the importance between non-canonical TCR-mediated activation pathway and MAP2K-mediated mechanism, which study in p38α-knock in mice ($Tyr^{323}$ was changed to phenylalanine (134). These mice are survival and have normal function, when knock in p38α genes, this result could suggest that a tissue-restricted was activated by Tyr323 phosphorylation (135). However, p38α activation in these mice (T-cells) is not depend TCR stimulation but based on phosphorylation of $Tyr^{323}$ (136).

In these knock mice, the mistake of p38α activation leads to delay in cell-cycle entry (136). In addition, the knock in T-lymphocytes (TCR-activated) could decrease IFNγ (interferon γ) production (136). From this, it could suggest that the p38α activation in Tyr323-mediated non-canonical pathway is involve in TCR stimulation (136). Moreover, TAK1-binding protein 1(TAB1) that can bind to p38α for activation, is caused of inducing p38α autophosphorylation in the stimulation loop (137, 138). The purify proteins of interaction between p38α and TAB1(for activation) has been difficult to reprint (139), even though it has been reported that the mechanism of TAB1 likely to involve in autophosphorylation, which may be cause of control in p38α activation during myocardial ischaemia and in some functions of myeloid cells (140-143). The alternative pathways of non-canonical MAP2K-independent mechanism for p38 MAPK activation have been demonstrated that in the Cdc7, protein kinase, could induce an abortive S-phase resulting in p38α-mediated apoptosis in HeLa cells (144). However, the information about the mechanism is unclear (144). The summary of mechanism in p38-MAPK activation has been show in Figure 5.

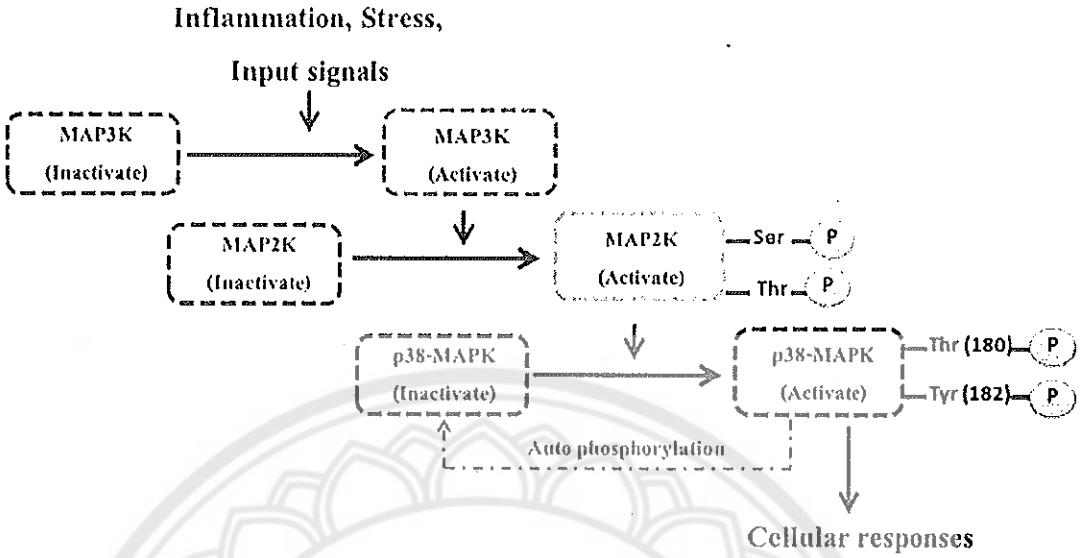


Figure 5 Mechanisms of p38 MAPK activation. The inflammation and stress can activate p38 MAPK by stimulated the series of MAP3K, inactivate, to MAP3K, activate, then the activated MAP3K can activate MAP2K by phosphorylation. After that, MAP2K can activate p38 MAPK result in p38 MAPK stimulated cellular responses. Moreover, p38 MAPK can autophosphorylation itself for activation of cellular responses.

3.2 p38 MAPK in myocardial ischemia

Myocardial ischemia is a strong stimulant in p38 MAPK activation that act as important pro-apoptotic kinase in cardiomyocytes (145). It have been demonstrated in preclinical investigations that the infarction/death and inhibits the production of inflammatory cytokines (aggravate ischemia injury), such as TNFα, interleukin-1 (IL-1), and IL-8 were decreased by in inhibition of p38 MAPK during prolonged ischemia (146, 147). In the term of reperfusion, a condition of re-occults of the coronary artery, could reduce ischemic myocardial injury (145). However, reperfusion could cause of generation a reactive oxygen species (ROS) and osmotic stress by p38 MAPK activation (145). Although, the evidence of myocardial injury and dysfunction that cause by p38 MAPK activation following ischemia/reperfusion is still studying and evolving (145, 148-150). But in many studies have been reported that the p38 MAPK activation, it was occurred in apoptosis and inflammation mechanism, might lead to ischemia/reperfusion injury (145). The first evidence of the p38α activation and p38β activation in the heart, which response to ischemia/reperfusion, was reported by Bogoyevitch et al. (151). In the later studies, which using ectopic gene expression, have been demonstrated that the cardiomyocyte apoptosis is involves in α isoform and this isoform alone could enough cause of cell death after ischemia (10, 11, 151, 152).

3.3 p38 MAPK inhibitor (SB203580)

The p38 MAPK inhibitor is many types classified according to the effect and functions including SB203580, which the first generation inhibitor, SD282 (an indole-5-carboxamide), VX745, SCIO469, SD0006 (a diarylpyrazole), SB681323 (dilmapimod), PH797804 (identified from a series of N-aryl pyridinones), BMS582949, R1503 and AW814141 (153). In addition, the SB706504 and SB 239063 are used as the p38 MAPK inhibitor in the inflammation studies (154, 155). The SB203580 (p38 MAPK inhibitor) is widely used in many studies of myocardial ischemia/reperfusion condition either *in vitro* model, a cardiac cell line or isolated cardiomyocytes from many species, or an *ex vivo* model or an *in vivo* model, and these finding demonstrated that the p38 MAPK inhibitor can usefulness in therapeutic of myocardial ischemia/reperfusion condition, including improved cardiac function,

inhibited infarct during expansion, reduced scar size and suppressed myocardial fibrosis (6). (Figure 6)

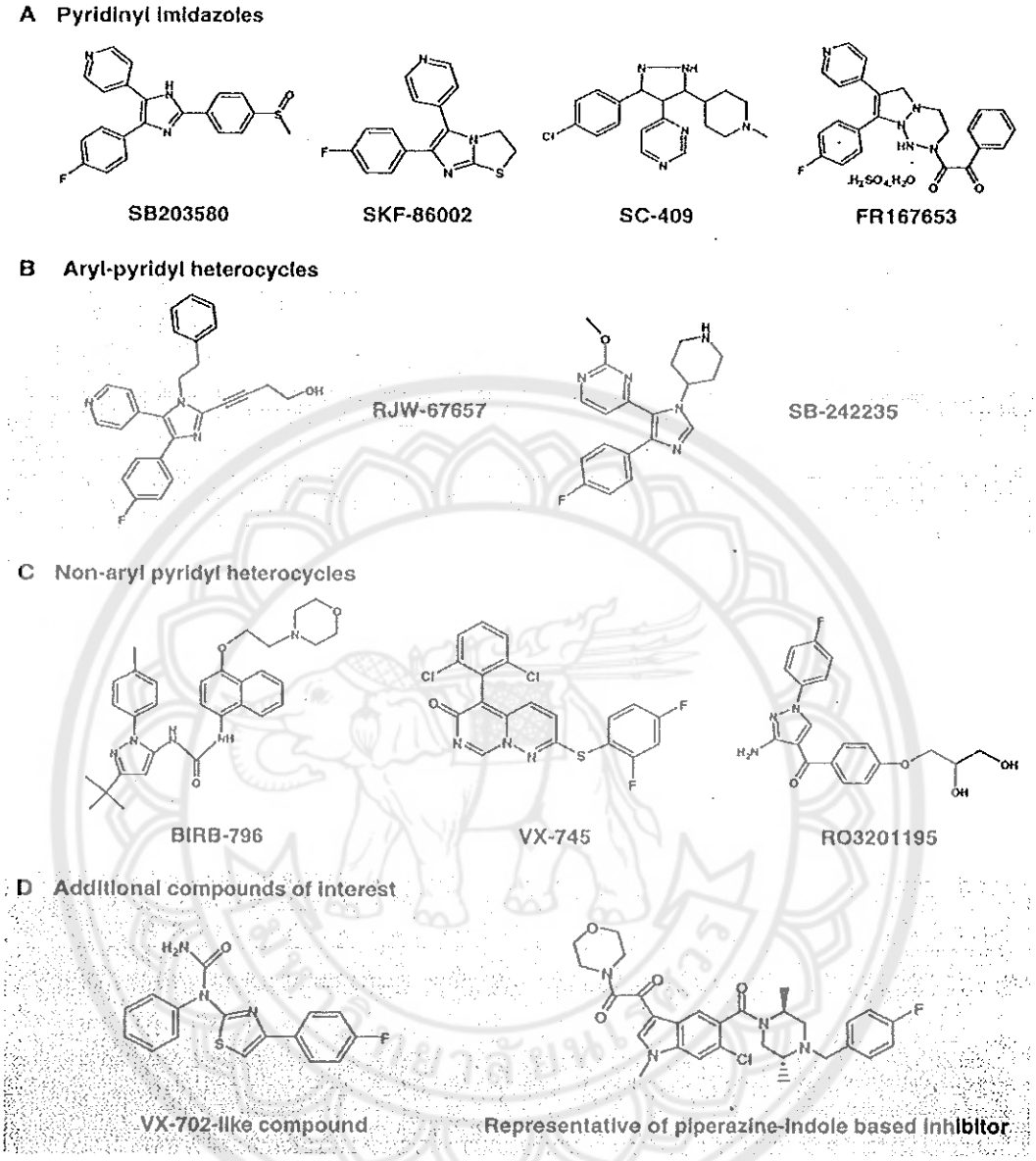


Figure 6 Structure of representative classes of p38 MAPK inhibitors. p38 inhibitors can be divided into 2 groups dependent upon their mode of binding to p38; active site inhibitors, such as SB203580 and RJW-67657, bind competitively to the ATP site of the enzyme whereas others bind remotely and interfere with ATP binding indirectly (such as BIRB-796). (146)

**Activation of p38 MAPK in insulin resistant and diabetic heart**

It has been reported that the worsening lethal injury during myocardial ischemia/reperfusion has been caused by the activation of p38 MAPK, and this injury or death can reduce by using pharmacological inhibitors for inhibition of p38 MAPK activity (6-11). Recently, it have been the information shown that the increased p38 MAPK activation in myocardial has strongly associated with insulin resistance and diabetes (156). It is well known that insulin resistant condition has a several risk factors including obesity, aging, inappropriate lifestyle, chronic inflammation combine with increased plasma insulin or free fatty acid levels (98, 157). Remarkably, the p38-MAPK is activated, when the early of insulin resistance condition was occurred (156). In cellular level, the alterations of cellular and biochemical during the progression to T2D, which is efficient of p38 MAPK activation has been described in Figure 7.

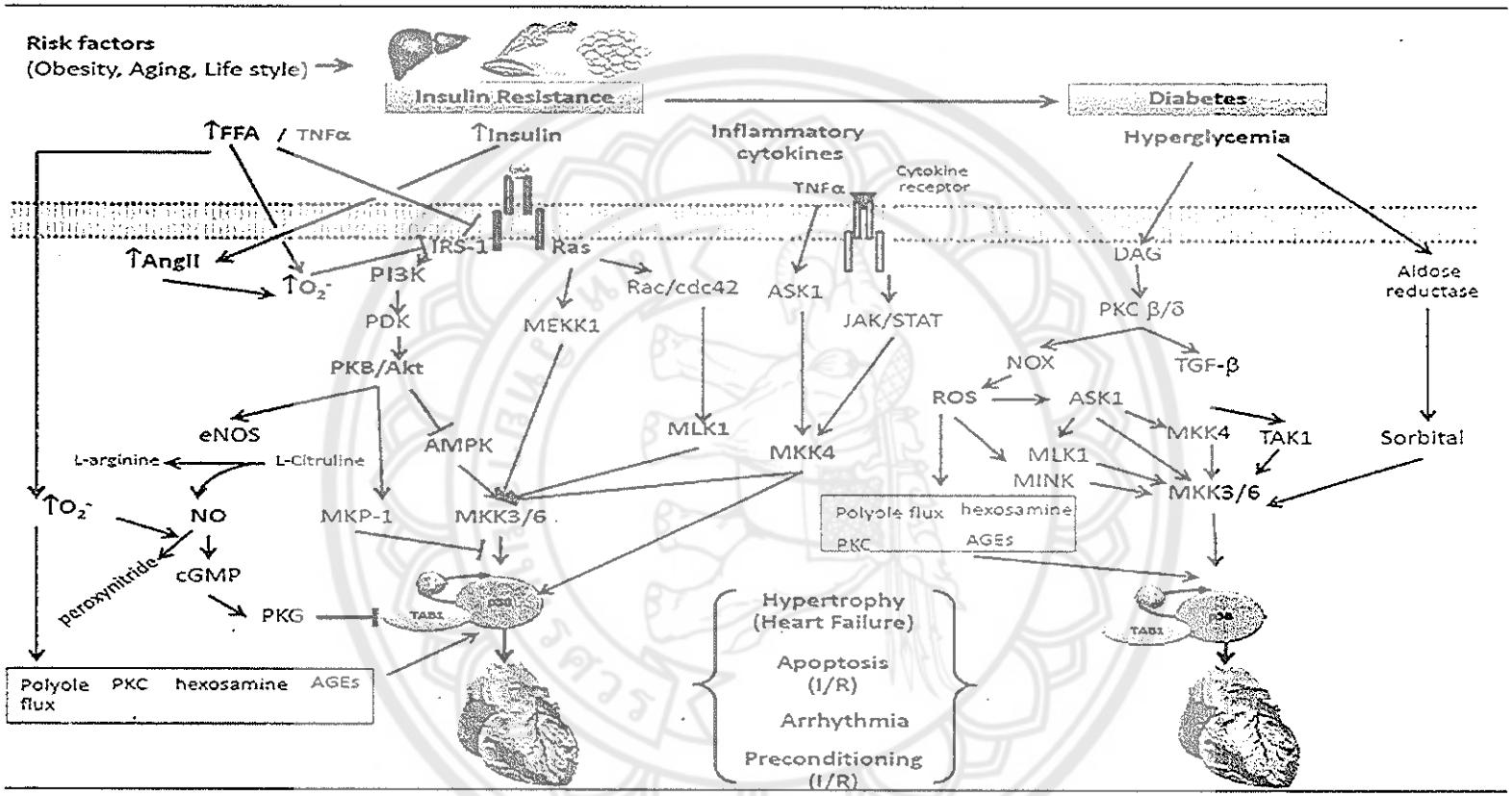


Figure 7 The signal transduction of activating p38 MAPK in insulin resistance and type 2 diabetes. In molecular level, the insulin resistance and type 2 diabetes can increase activation of p38 MAPK many pathway including reducing sensitivity of insulin receptor substrate-1 by increased FFA and $O_2^-$. (21)

The insulin resistance condition is a condition of worsening downstream of insulin receptor in insulin signalling pathway, which it is the signalling via the pathway of phosphatidylinositide-3 kinase (PI3K)/Protein Kinase B (PKB, or Akt) (158).

In normal heart, the cardiac growth, metabolism, and functions was controlled or sustained by the PI3K-Akt signalling cascade that equivalence with the classical mitogenic cascade (Ras-ERK) co-operatively (159). Whereas, in the insulin resistance heart has been shown the involve in the PI3K-Akt cascade, rather than the Ras-ERK cascade, by reduced sensitivity the insulin signalling (159).

Moreover, the impairment of insulin signalling (in insulin resistance) and responses due to the increasing of the plasma free fatty acid (FFA), from this cause of activating p38-MAPK in the heart. The mechanism in activation p38 MAPK of FFA are described as, the increased FFA can block the signalling of insulin receptor substrate-1 (IRS-1) that activate to the intracellular section of insulin receptor that consequence to reduce the PI3K-Akt activation (160, 161). In normally, the inactivation of AMPK (The upstream activator of MKK3/6-p38-MAPK) was occurred by the phosphorylation AMP-activated protein kinase (AMPK) of activated PKB/Akt (141, 162, 163). Therefore, the decreasing of activating PI3K-Akt pathway in insulin resistance could cause of indirect p38-MAPK activation (Figure 8). Besides, the activation p38 MAPK of PI3K-Akt signalling not only through phosphorylate AMP-activated protein kinase (AMPK), but also decrease the MAPK phosphatase-1 (MKP-1) activity, phosphatase activity, that result in p38 MAPK activation (164, 165). Interestingly, another part of the worsening of PI3K-Akt activation also lead to activate the endothelial nitric oxide synthase (eNOS) phosphorylation and hyperglycaemia-induced eNOS glycosylation by O-GlcNAc modification, from this activation result in reducing of the nitric oxide (NO) production (159). After that, this NO could activate cGMP and protein kinase G-I (PKG I), leading to autophosphorylation of p38 MAPK, which result in interfere with TAB1 (stimulator of autophosphorylation in p38 MAPK) (166), and the impaired PI3K-Akt activation leads to reduce in NO could increase p38 MAPK activation (Figure 7).

The high rate of myocardial fatty acid uptake and oxidation were induced by the high concentration of plasma FFA, and in cardiomyocytes primarily the β-oxidation of fatty acid was occurred in the mitochondria and lesser extent in myocardial peroxisomes (167, 168), where the ROS production was occurred , which as well as the

locate of uncoupling oxidative phosphorylation (169, 170). The ROS production and oxidative stress that was increased, lead to stress-sensitive serine/threonine kinase signalling activation, by decreasing of tyrosine phosphorylation that cause of insulin stimulation and result in increasing phosphorylated IR and IRS protein at the serine and threonine residues (171, 172). Moreover, it has been shown that FFA could induce oxidative stress and reduces the intracellular antioxidant molecules, for example the glutathione (173, 174). This phenomenon aggravates the insulin signalling impairment and worsens insulin resistance.

The mitochondrial superoxide that overproduction can causes of increasing produce in the advanced glycation end products (AGEs), polyole pathway flux, the protein kinase C (PKC) isoforms activation and enhance the activity of hexosamine pathway (175). These metabolic products could cause of p38-MAPK activation (176-180) (Figure 8). Moreover, a high amount of insulin, which produced by the pancreatic cells, when it under an insulin resistant condition. From this, leading to the hyperinsulinemia condition that was occurred in insulin resistance. Finally, cause of a strongly stimulated in a classical Ras-MEKK1signaling pathway, and it may be indirect the MKK3/6-p38 MAPK activation (181, 182). Furthermore, the activation of MLK, which is an upstream kinase activated of MKK3/6–p38-MAPK cascade, cause by the Ras phosphorylation result in the Rac/cdc42 stimulation (183, 184). From this, leading to increased p38 MAPK activation (Figure 7).

In addition, hyperglycaemia not only activate Ras-MEKK1signaling pathway, but also increase the plasma angiotensin II (Ang II) level, an octapeptide that is a potent vasopressor, through the hyperinsulin- induced Renin-Angiotensin system (185). From this, the superoxide that produced by the increasing of Ang II level and it can mediate in the changing between NO into peroxynitrite and leading to reduce the NO bioavailability (186), that it can inhibit the NO-cGMP-PKG I pathway and promote TAB1-induced p38-MAPK autophosphorylation (Figure 7).

The insulin resistant condition could secret the inflammatory cytokines such as TNF-α, IL-1, IL-18, IL-6, monocyte chemotactic protein (MCP)-1 and C-reactive protein (CRP) (187-192). It have been reported these cytokines can activate p38-MAPK through either the JAK/STAT-MKK4 pathway or the apoptosis signal-regulating kinase 1 (ASK1) pathway (112). It has been shown that TNF-α could reduce tyrosine

phosphorylation of IRS-1 and increase induce serine phosphorylation, result in the worsening of the IRS-1/PI3K/Akt pathway (193). In addition, decreasing of Akt phosphorylation in this situation could attenuate NO generation that can cause of p38-MAPK autophosphorylation, which induced by TAB1 as previously discussed, lead to increase p38-MAPK activation (166).

The insulin resistance can develop to type 2 diabetes characterized, which it previously mentioned, by the hyperglycaemic condition and it has evidence shown to cause the generation of sorbitol by activate the aldose reductase activity(194-196), which is known as a strongly activator of p38 MAPK (Figure 8) (197). Furthermore, the plasma glucose level was increased also increases de novo synthesis of diacylglycerol (DAG), then consequence to activates protein kinase C (PKC) (198). This activate of PKC is an important signal transduction that it activator of the transforming growth factor beta 1 (TGF-β) activation (176), which afterward activates the MKK3/6-p38-MAPK cascade (Figure 7).

It is well known that the hyperglycaemia condition is cause increased product of reactive oxygen species (ROS) through mitochondrial ROS generation pathway, as previously illustrated that could result in activation of NAD(P)H oxidase (NOX) pathway (199) . From this, the NOX, which received from ROS, can cause of activation in various redox-sensitive kinases including Misshapen/NIKs-related kinase (MINK) (200), finally result in the MKK3/6-p38 MAPK pathway activation (201). Moreover, NOX can generate further amounts of ROS, especially superoxide anion ($O_2^-$) and could enhance the overall oxidative stress. The $O_2^-$ level that increasing could also cause of the generation of advanced glycation end products (AGEs), polyole pathway flux, protein kinase C (PKC) isoforms activation and the activity of hexosamine pathway was increased (Figure 7), which could activate p38-MAPK, as previously mentioned. The high level of superoxide ion ($O_2^-$) caused of the reduction in NO bioavailability (202), resulting in p38 MAPK autophosphorylation that induced by TAB1, and finally cause of activating p38 MAPK (Figure 7). In addition, the high plasma glucose can activate apoptosis signal-regulating kinase 1 (ASK1) (203), which is the MAPKK kinase capable of duplex activating JNK and p38 MAPK via MKK4 and MKK3/6, or mixed linage kinase 1(MLK) 1 (Figure 7) (204).

It have been demonstrated that the myocardial p38-MAPK activation is plays an important role in the pathophysiological mechanism of cardiomyopathies such as ischemia-induced cardiomyocyte injury and apoptosis (157), cardiac arrhythmia (205) and cardiac hypertrophy (206). Since several biochemical changes occurring could result in an activation of p38 MAPK in the diabetic heart condition, this association may be a mechanistic elucidation of cardiac complications that found in diabetic subjects (207-213). Therefore, the p38 MAPK activation was inhibited by p38 MAPK inhibitor in insulin resistance and diabetes may be a hopeful therapeutic process to protect or reduce early cardiac complications in diabetic patients.

**Effect of metformin on myocardial p38 MAPK signalling**

The Bax protein is activated by p38 MAPK in ischemic reperfusion injury and AMPK, respectively (214, 215). This experiment also determined the effect of AICAR treatment, which an AMP analogue, for confirming (216). It has been shown that the effect of metformin to activate p38 MAPK on isolated rat ventricular myocytes, leading to increased cardiac cell death (111). Whereas, the treatment of metformin could show the cardioprotective effect of the drugs in the rat cardiac myoblast cell line (H9c2) that has the activation of p38 MAPK (110). Moreover, it has been reported in *ex vivo* model study that the treatment of metformin in perfusion isolated rat heart can increase cardiac output through activation of p38 MAPK (110). It has been reported the activated p38 MAPK that treated with metformin can stimulate the IL-6, which has cardioprotective effect (217), for inhibition of apoptosis condition (109). The summarized effects of metformin on p38 MAPK activation were shown in Figure 8.

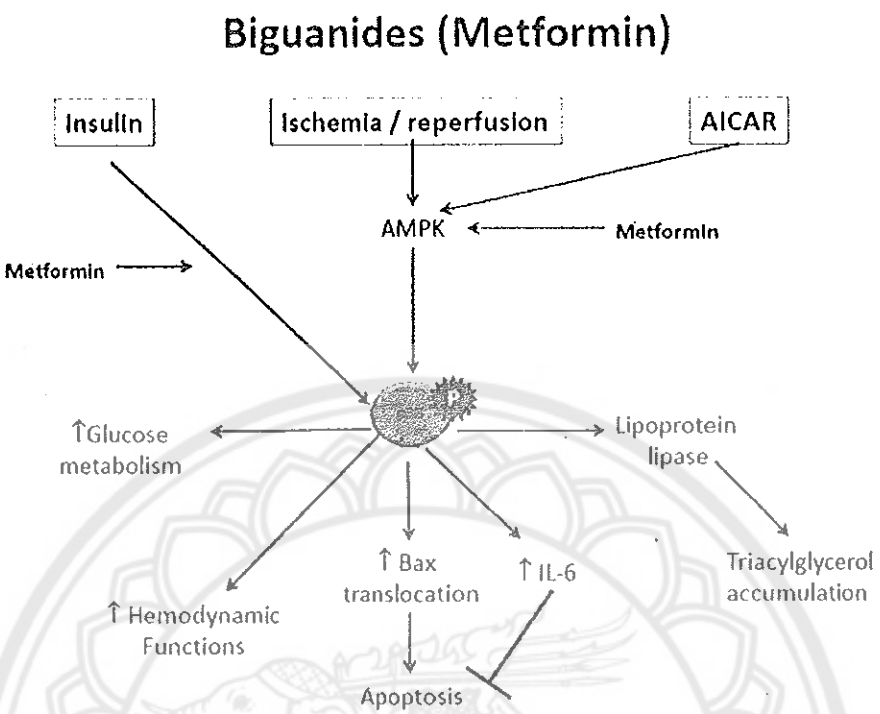


**Figure 8 Effect of metformin (biguanides drugs) on activating p38 MAPK in the insulin resistant heart. The biguanides (metformin) can improve apoptosis in heart with ischemia/reperfusion by activate IL-6. Moreover, it can increase glucose metabolism. (21)**

**Effect of p38 MAPK inhibitor on insulin resistance and diabetes**

It is known as the pathophysiology in cardiovascular dysfunction is involved to activate p38 MAPK (6, 218-221). So, the inhibition of p38 MAPK activation, which one of the therapeutic potential in this disease is suggested (6, 222-224). The important information that has been found in insulin resistance condition and diabetic models, demonstrated the activated p38 MAPK could worsen in cardiomyopathy (156). Therefore, it may be helpful and improve in the heart with insulin resistance condition, when inhibit the p38 MAPK activation by pharmacological inhibitors. It have been evidence reported that the treatment of insulin resistant or diabetic model by using the p38-MAPK inhibitors could improve left ventricular function, endothelial function and reduce cardiac inflammation (225). Moreover, evidence demonstrated that the administration of p38 MAPK inhibitor, SB203580, can help the cardiac contractile functions in insulin resistance condition and hyperleptinemia (17), and also cause of the inhibition in advanced glycation end products (AGEs), cause of overexpression of collagen type I production in cardiac fibroblasts, which were involved in cardiac fibrosis (226). This evidence suggests the benefit of p38 MAPK inhibition in insulin resistance or diabetes. Recently, it has been reported about the cardioprotective effects of the p38 MAPK inhibitor, SB203580, in an ischemic/reperfusion injury in rats (205). However, information in regard to p38 MAPK inhibition in ischemia/reperfusion injury of the diabetic heart has never been investigated.

# CHAPTER III

# RESEARCH METHODOLOGY

This chapter mention about the methodology of this research including material apparatus and methods. The information of each topic is explained below.

## Research methodology

This thesis is divided into 3 major parts. The *in vitro* experiments were performed by using rat cardiac myoblast cell (H9c2 cell), the *in vivo* treatment of drug and the *ex vivo* experiment on cardiopathophysiology were performed in animal model (the male Wistar rats and Goto-Kakizaki (GK) rats).

The first part *in vitro* experiments, was divided into 2 parts, the hyperglycaemia group and the hyperglycaemia subjected to simulated ischemia/reperfusion injury group. In the first part, the H9c2 cell was induced to hyperglycaemia by hyperglycaemic solution (high-glucose solution). After that, the induced cell was treated with anti-diabetic drug metformin, or p38 MAPK inhibitor SB203580, or combination of metformin and SB203580. Then, the treated cell was measured cell survival by MTT viability assay, reactive oxygen species (ROS) production by 2′-7′-Dichlorodihydrofluorescein (DCFH-DA) staining and the signal transduction by Western blot analysis. In second part, the H9c2 cells were also incubated in hyperglycaemic condition. Then, the induced cell was treated with metformin, SB203580, or combination of metformin and SB203580. After that, the treated cell was subjected to simulated ischemia/reperfusion injury (sI/R; *in vitro*) by using ischemic buffer. Finally, cell death and the signalling in response to the combination drugs were determined by MTT assay and Western blot analysis, respectively.

The second part was performed in an *in vivo* experiment used the male Wistar rats and male GK rats. The experimental design was divided into 2 groups; the control group and the diabetes group. All groups were fed with normal diet and normal drinking water. Then, the rats were measured fasting plasma glucose (FPG), oral glucose tolerance test (OGTT) and glycated haemoglobin test ($HbA_{1c}$ test) for diagnosis diabetic condition. When the rats were diagnosed with diabetic condition, then it was treated

with anti-diabetic drug metformin, or p38 MAPK inhibitor SB203580, or combination of metformin and SB203580. After that all groups were used for further experiments.

The last part was performed in an *ex vivo* experiment using the male Wistar rats and male GK rats. The experimental design was also divided into 2 groups; the control group and the diabetes group. All groups were fed with food and drinking water, as mentioned above. Then, the rats were treated with anti-diabetic drug (Metformin) or p38 MAPK inhibitor SB203580, or combination of metformin and SB203580. After treatment, the rats were subjected to an *ex vivo* global ischemia/reperfusion injury condition by Langendorff perfusion system. Finally, all groups were measured for biochemical parameters including plasma glucose, plasma insulin, the cardiac function and infarct size. Moreover, the signal transduction in response to combination drugs signalling in organ level was determined. (Figure 9)

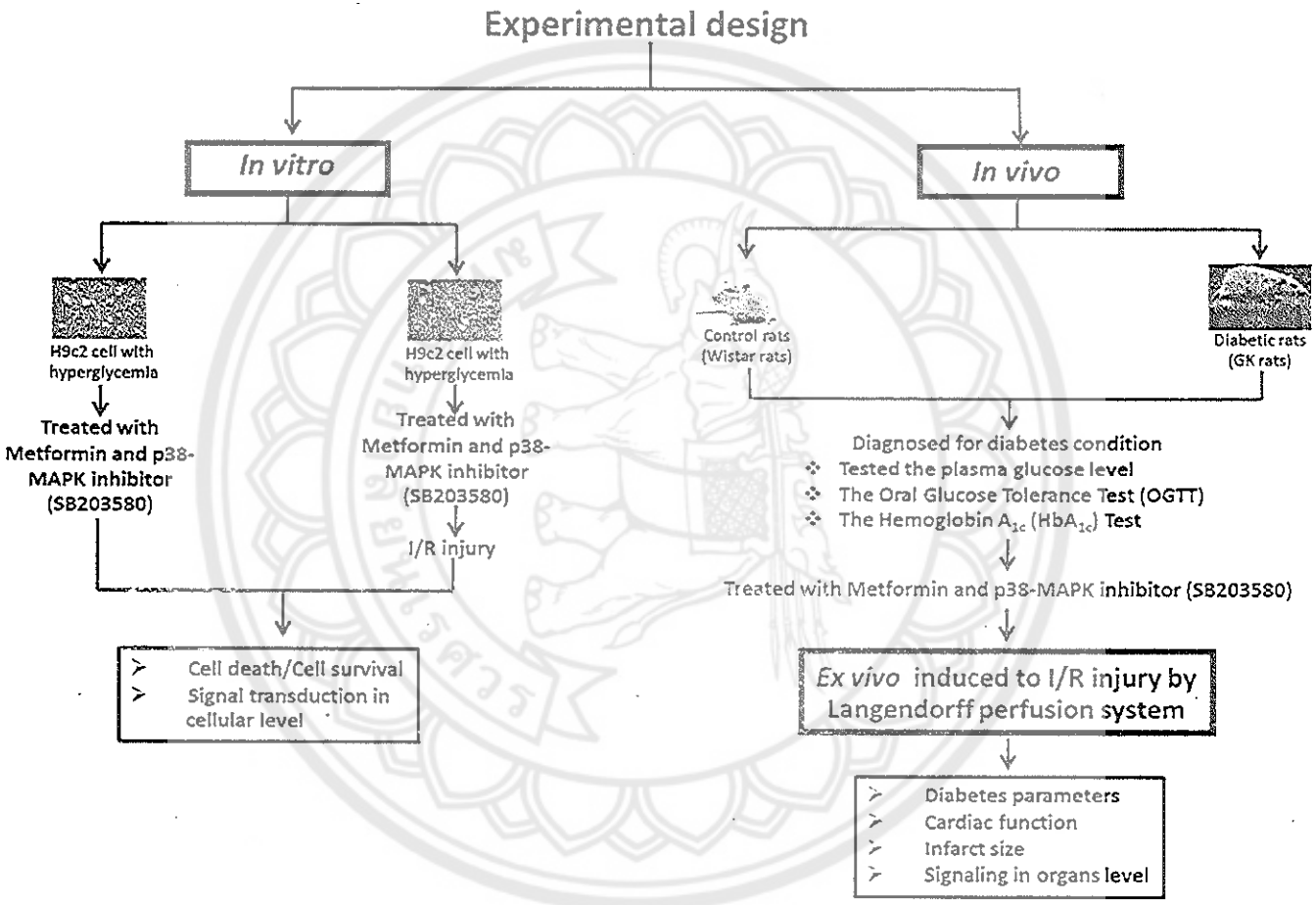


**Figure 9 The experimental design and methods.**

**Cell type and cell culture (*In vitro* experiments)**

**1. Rat cardiac myoblast (H9c2) cell line culture**

1.1 Origin of H9c2 cell line

The H9c2 cell was constructed in 1976 by Kimes and Brandt (227). This cell is derived from embryonic BDIX rat heart tissue and shown the feature of skeletal muscle. In addition, it could express nicotinic receptors and generate phosphokinase isoenzyme (227). Moreover, Hescheler and colleagues reported that the H9c2 cell showed morphological characteristics similar to cardiac myoblast, in addition, it also showed the biological properties of adult cardiac cells (228). Therefore, this cell line appropriate as an *in vitro* model for cardiac muscle. The H9c2 cell used in this study was purchased from American Type Cell Culture (ATCC-CRL1446).

1.2 Cell culture/sub-culture

H9c2 cell line were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco®) supplemented with 5000 units/ml of penicillin, 5000 µg/ml of streptomycin (Gibco®) and 10% Fetal Bovine Serum (FBS) (Gibco®). Cells were cultured at 37°C, 5% carbon dioxide ($CO_2$) and 95% oxygen ($O_2$) throughout the experiment, except in some tested experiments.

When the cells density is more than 70-80% confluent, the cells were sub-cultured by discarding the culture medium. Then, the cells were added with 5 ml pre-warmed phosphate buffer saline (PBS) for washing twice. After that, 1 ml of pre-warmed trypsin-EDTA (Gibco®) was added and incubated at 37°C for approximately 2 min or when observe under microscope the cell detached from the flask surface. Then, the trypsin activity was terminated by adding 5 ml of pre-warmed completed DMEM. The cell suspension was transferred into 15 ml sterile tube and centrifuged at 1,800 rpm for 10 min. The supernatant was discarded, and the cell pellet was resuspended with 1 ml of completed DMEM. The cells were transferred into a new cell culture flask and the flask was added at least 4 ml of completed DMEM.

**2. *In vitro* hyperglycaemic condition.**

2.1 Induced hyperglycaemic condition

The H9c2 cell was stimulated to hyperglycaemic condition. The cell was cultured in DMEM supplemented with 10% FBS and was transferred to 96-well plate at a concentration of 1 x $10^5$ cell/ml and cultured until reach 60-80% confluence.

Then, cell was exposed to 5.5 mM of D-glucose in complete medium as control group and other groups were exposed to various concentration of D-glucose (11, 22, 33 and 60 mM) in complete medium as hyperglycaemia groups (229). After that, all groups were cultured at 37°C, 5% carbon dioxide ($CO_2$) and 95% oxygen ($O_2$) for 24 or 48 or 72 or 96 h (229). After incubation, cell survival was measured by MTT assay. Moreover, to determine the hyperosmolar effect, the culture sugar osmolality groups were performed by using D-mannitol. The H9c2 cell was cultured in DMEM complete medium supplemented with D-mannitol (5.5, 11, 22, 33 and 60 mM) for 24 or 48 or 72 or 96 h, which correspond to concentration and time used in D-glucose. At the end of incubation, cell viability was also measured by MTT viability assay.

2.2 The effect of combination between metformin and p38-MAPK inhibitor (SB203580) on H9c2 cell induced to hyperglycaemic condition.

The induced hyperglycaemia cell, the cell from 1.2.1, was divided into 2 groups including control group and hyperglycaemic group. The control group was divided into 4 subgroups and the hyperglycaemic group was divided in to 4 subgroups (Table 3). After that, all groups of cells were cultured at 37 °C, 5% carbon dioxide ($CO_2$) and 95% oxygen ($O_2$) for 24 h. Finally, the cell viability was measured by MTT viability assay.

**Table 3 Condition of experimental design groups in in vitro hyperglycaemic condition**

| Groups | Conditions |
|---|---|
| Control | H9c2 cell in complete medium |
| | H9c2 cell in complete medium + 3 mM of Metformin |
| | H9c2 cell in complete medium + 10 μM of p38-MAPK inhibitor (SB203580) |
| | H9c2 cell in complete medium + 3 mM of Metformin + 10 μM of p38-MAPK inhibitor (SB203580) |
| Hyperglycae-mia | H9c2 cell + D-glucose at optimal concentration that received from previous experiment (Hyperglycaemia) |
| | Hyperglycaemia + 3 mM of Metformin |
| | Hyperglycaemia + 10 μM of p38-MAPK inhibitor (SB203580) |
| | Hyperglycaemia + 3 mM of Metformin + 10 μM of p38-MAPK inhibitor (SB203580). |

**3. *In vitro* the hyperglycaemia subjected to ischemia/reperfusion injury condition.**

3.1 Optimization of simulated to Ischemia/Reperfusion injury timing

The H9c2 cell line was maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco®) supplemented with 5000 units/ml of penicillin, 5000 µg/ml of streptomycin (Gibco®) and 10% Fetal Bovine Serum (FBS) (Gibco®) in 96 well plate. When cell populations reached 80% confluence, the cell was washed with PBS. For simulated ischemia, H9c2 cell was induced by treatment with ischemic buffer [10X stock basic buffer (137 mM NaCl, 3.58 mM KCl, 0.49 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 4 mM HEPES), sterile $ddH_2O$, 20 mM 2-deoxyglucose, 30% Na lactate, 1.0 mM Na dithionite] for 20, 40, 60, 120 and 180 min at 37°C. After that, ischemic buffer was discharged and 150 µl complete DMEM medium was added for reperfusion for 24 h at 37°C, 5% $CO_2$. After that, the cell viability was measured by MTT viability assay, for optimal suitable time to cause of 50% cell death.

3.2 The effects of combination between metformin and p38-MAPK inhibitor (SB203580) in H9c2 cell induced to hyperglycaemia subjected to ischemia/reperfusion injury condition.

The induced hyperglycaemia cell, from 1.2.1, was treated with the combination between 3 mM of metformin and 10 µM of p38-MAPK inhibitor (SB203580). After that, the treated cells were divided into 2 groups, the control group was divided into 5 subgroups and the induced hyperglycaemic subjected to ischemia/reperfusion group was divided into 5 subgroups (Table 4). At the end of protocol, the treated cell was measured by MTT viability assay.

**Table 4 Condition of experimental design groups in in vitro the hyperglycaemia subjected to ischemia/reperfusion injury condition**

| Groups | Conditions |
|---|---|
| Control | H9c2 cell in complete medium |
| | H9c2 cell in complete medium + 3 mM of Metformin |
| | H9c2 cell in complete medium + 10 μM of p38-MAPK inhibitor (SB203580) |
| | H9c2 cell in complete medium + 3 mM of Metformin + 10 μM of p38-MAPK inhibitor (SB203580) |
| | H9c2 cell in complete medium + simulated to ischemia/reperfusion (SI) |
| Induced hyperglycaem-ic subjected to ischemia/repe-rfusion | H9c2 cell + D-glucose at optimal concentration (hyperglycaemia) |
| | H9c2 cell + hyperglycaemia + SI |
| | H9c2 cell + hyperglycaemia + SI + 3 mM of Metformin |
| | H9c2 cell + hyperglycaemia + SI + 10 μM of p38-MAPK inhibitor (SB203580) |
| | H9c2 cell + hyperglycaemia + SI + 3 mM of Metformin + 10 μM of p38-MAPK inhibitor (SB203580) |

**4. Measurement of cell viability assay**

Cell viability was assessed by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT viability assay). The culture medium was removed and 0.5 mg/ml MTT reagent was added for 2 h and incubated at 37°C. After incubation, MTT reagent was discarded and the formazan dye was solubilized by DMSO. The optical density (OD) was determined by spectrophotometer at 490 nm using DMSO as a blank. Finally, the difference in cell death between control and treated cells were calculated as percentage.

**5. Determination of cellular reactive oxygen species (ROS) of the hyperglycaemia condition**

2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining is one of the most widely techniques that used for assessing the ROS produced by the cell. The H9c2 cell was cultured in DMEM supplemented with 10% FBS and was transferred to 96-well plate at a concentration of 1 x $10^5$ cell/ml and cultured until reach 60-80% confluence. Then, the cell was incubated with 25 µM of DCFHDA solution that contain in 33 mM glucose solution or 33 mM glucose solution + 3 mM metformin or 33 mM glucose solution + 10 µM SB203580 or 33 mM glucose solution + 3 mM metformin + 10 µM SB203580 in the dark in the dark at 37°C for 24 h. After incubation, the ROS activity was measured the fluorescence intensity by using the EnSpire Multimode Plate Readers (PerkinElmer, Massachusetts, USA). The filter suitable for detecting the signal gave the excitation wavelength at 498 nm and emission wavelength at 522 nm.

**6. Determination of cellular signalling by Western blotting**

6.1 Preparation of protein sample

Cells culture from each experimental group were lysed by 200 µl of 2X SDS-sample buffer, containing 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol in 300 mM Tris-HCl, pH 6.8. Then, extracted protein was transferred to the new pre-cooled microcentrifuge tube. The sample was boiled for 10 min and stored at -80°C until analysis. The protein lysates were separated on 12 % (v/v) SDS–polyacrylamide gels electrophoresis at 120 volts and were transferred to Polyvinylidene fluoride (PVDF) membranes (Hybond-P, GE Health Care) using semi-dry transfer, which was mentioned in 6.2.

6.2 Protein transfer

The separated proteins on polyacrylamide gel were transferred to PVDF membrane by using 15 volts of semi-dry electroblotting (Biorad) for 60 min. The transferred protein on PVDF membrane was incubated for 1 h with 5% (w/v) skim milk in Tris- buffered saline (pH 7.4) containing 0.1% (v/v) Triton X-100 (TBST), for avoiding nonspecific binding, and probed with the appropriate primary antibodies recognizing the interesting protein such as phosphorylated-Akt (Santa cruz biotec #sc-7985), total-Akt (Santa cruz biotec #sc-8312), phosphorylated p38 (Santa cruz biotec #sc-17852) and total-p38 (Santa cruz biotec #sc-535) (All of primary antibodies were diluted at 1:500 (except p38 was diluted at 1:1,000) in 1% skim milk + TBST buffer) for overnight at 4°C. Then, the membrane was washed and exposure to horseradish peroxidase-conjugated secondary antibody, which diluted at 1: 2,000 in 1% (w/v) skim milk + TBST, for 1 h at room temperature. After that, the membrane was developed using an enhanced chemiluminescence substrate (Amersham, England). Bands corresponding to the protein of interest were appeared and the intensity of each band was measured by Chemidoc™ XRS+ Imaging System with Image Lab ™ 2.0 Software (BIORAD). Comparison of the band intensity between lanes will provide information on relative abundance of the protein of interest.

**Animal experiments**

Type 2 diabetes is a non-communicable disease that causes major global health problems. It has been predicted to cause of double death rate between 2005 and 2030 (3). Therefore, development of new strategy for diabetic treatment could decrease risk of diabetes prevalence. Most of diabetic studies have been performed in animal models including obese models and non-obese models (230). Usually the major cause of diabetes associated with obesity. However, it has been several reported that mostly Asian population, which is non-obesity, has high prevalence in type 2 diabetes (231-233). So, in this study was performed in GK rats, which is a non-obese diabetic model, for mimic type 2 diabetic disease.

Goto-Kakizaki (GK) rats is spontaneous type 2 diabetes non-obese model. They are established by Goto and colleague in Japan in 1973. It is generated from selective inbreeding of Wistar rats with abnormal glucose tolerance repeated over

several generations. The characteristic of GK rats are non-obesity, middling and stable fasting hyperglycaemia, hypoinsulinemia, normolipidemic and an early onset of diabetic complications (230). The GK rats have been presented an impaired glucose tolerance at 2 weeks of age, moderate insulin resistance and mild hyperglycaemia after weaning (after 3 weeks of age) (230, 234). In addition, they are indicated several complications at 12 weeks of age including cardiovascular disease, abnormal in bone structure and damage to the kidney (234).

Animal in these experiments were managed by following WHO Guidelines for Breeding and Care of Laboratory Animal. The protocol is approved by the committee of Centre for Animal Research Naresuan University (NU-AE581023). All surgery was conducted under anaesthesia as described in the respective method parts and all efforts were made to minimize suffering.

**1. Animal model**

In this study using adult male Wistar rats and male GK rats (180-250 g, n = 10 per each group) that obtain from the Nomura Siam International Co. Ltd, Thailand. All animal was maintained under an environmentally control condition (22 ± 5°C, 12 h light/12 h dark cycle) at Centre for Animal Research Naresuan University, Naresuan University, Phitsanulok, Thailand. All of rats were fed with standard laboratory chow (4.02 kcal/g of total calorie constituent; Mouse Feed Food No.082, C.P. Company, Bangkok, Thailand: as shown in table 5). All animals in this protocol were approved by the committee of Centre for Animal Research Naresuan University (NU-AE581023).

**2. Diagnosis of insulin resistance and diabetic-like condition**

The male Wistar rats or the GK rats were divided into 2 groups. First group is a control group (C), which was performed in the Wistar rats. Second group is a diabetic group (D) that was performed in the GK rats. The body weight was measured every week throughout the experiment. Blood was collected from the tail vein at week 7 and week 14 for blood glucose analysis. The rats were diagnosed for diabetic condition by using the criteria of the plasma glucose level or the oral glucose tolerance test (OGTT) or the Haemoglobin $A_{1c}$ ($HbA_{1c}$) Test. (Table 6)

**3. Methods for analysis.**

3.1 Determination of blood glucose level

The blood samples were collected from tail vein for measuring of blood glucose level by glucometer (SD GlucoNavii® GDH). The rats were fasted for 12-14 h. Then, the blood sample was collected from tail vein by using 1 ml needle syringe. After that, the blood sample was dropped on glucose strip and measured by glucometer. Finally, the level of blood glucose was represented on LCD screen of glucometer. The method of glucose analysis is as according; the glucose-1-dehydrogenase (GDH) oxidizes glucose in blood samples into gluconolactone. Then, the FAD oxidized by gluconolactone into $FADH_2$. The $FADH_2$ exchanges electrons with the mediator and the mediator is oxidized into electricity. Finally, the amount of electricity is proportional to the glucose content in the sample.

3.2 Determination of plasma insulin level

The plasma insulin levels were determined by Sandwich ELISA (Millipore, MI, USA). The principle of the method is sequentially on capture of insulin molecules from samples to wells of a microtiter plate coated by monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin, after that wash away of unbound material from samples. Binding of horseradish peroxidase to the immobilized biotinylated antibodies, then wash away of free enzyme conjugated and quantification of immobilized antibody-enzyme conjugated by monitoring horseradish peroxidase activities in the substrate 3,3',5,5'-tetramethybenzidine. The enzyme activity was measured by a spectrophotometer (BioTek, Winooski, VT, USA) at absorbance 450 nm, corrected from the absorbance 590 nm. Since the increase in absorbency is directly proportion to the amount of capture insulin in the unknown samples, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standard of known concentration of rat insulin (235).

3.3 Determination of oral glucose tolerance test (OGTT)

An oral glucose tolerance test (OGTT) was performed in all of group before treatment for diabetic diagnosis. Rats were fasted for 12-14 h, and then the blood was collected from tail vein as baseline. After that, rats were fed with 2 g/kg body weight

of 40% (w/v) glucose solution by gavage. Blood samples were taken 30, 60, 90 and 120 min after the glucose meal and analysed for blood glucose with a glucometer (236, 237).

3.4 Determination of Haemoglobin $A_{1c}$ ($HbA_{1c}$)

The $HbA_{1c}$ was performed following CLOVER A1c™ Self-analyser (Fully automatic). The haemoglobin $A_{1c}$ level was measured by blood samples from tail vein. The blood sample was collected from tail vein by using 1 ml needle syringe. After that, the blood sample was dropped on test cartridge and measured by CLOVER $A_{1c}$™ Self system. Finally, the percentage of Haemoglobin $A_{1c}$ ($HbA_{1c}$ %) of blood sample was represented on LCD screen of CLOVER A1c™ Self machine. The principles of Haemoglobin $A_{1c}$ analysis is as according; the test cartridge is containing the reagents (a cartridge and a reagent pack) necessary for measurement of haemoglobin $A_{1c}$. After, the blood sample was dropped on cartridge. The red blood cell was lysed by the reagent pack, which composed of reaction solution and washing solution. Then, the cis-diols of glycated haemoglobin was bound by boronate resin and the non- specific glycated haemoglobin was eliminated by the washing solution. Finally, the amount of glycated haemoglobin was measured by photometrical.

**4. Drugs treatment in animal model.**

All of rats were divided into 5 groups, after it was diagnosed as diabetes, (Figure 10) including control group (Wistar rat; treated with deionized (DI) water), diabetes control group (GK rat; treated with DI water), diabetes treated with metformin group (GK rat; treated with Glucophage, Merck Serono, Thailand; 15 mg/kg.BW, b.i.d (238, 239)), diabetes treated with P38-MAPK inhibitor (SB203580) group (GK rat; treated with p38-MAPK inhibitor, 2 mg/kg.BW, Tocris Bioscience, MI, USA (240) and diabetes treated with metformin and p38-MAPK inhibitor (SB203580) group (GK rat; treated with Glucophage, Merck Serono, Thailand; 15 mg/kg.BW, b.i.d (238, 239) combined with p38-MAPK, inhibitor, 2 mg/kg.BW, Tocris Bioscience, MI, USA (240). The rats in each group were treated for 4 weeks (238, 239) and then, the blood was collected from tail vein every week for determining blood glucose. At the end of the experimental period, rats were subjected to ischemia/reperfusion study (*Ex vivo*).

**5. Determination of cardiac function by Echocardiography**

All of rats were intraperitoneal injected (IP) with Ketamine/xylazine cocktail (91 mg/kg Ketamine; 9.1 mg/kg Xylazine) at a concentration of 0.1 ml/100 g (v/w) for anaesthetic. Then the chest hair was removed and measured the cardiac function parameters including Ejection fraction (EF), End-Diastolic volume (EDV), End-Systolic volume (ESV), Interventricular septum thickness at end-diastole (IVSd), Interventricular septum thickness at end-systole (IVSs), Left ventricular internal dimension at end-diastole (LVIDd), Left ventricular internal dimension at end-systole (LVIDs), Left ventricular posterior wall thickness at end-diastole (LVPWd), Left ventricular posterior wall thickness at end-systole (LVPWs), Cardiac output (CO), Stock volume (SV) and Heart rate (HR) by using Mindray M9 (Mindray Medical Co., Ltd., Thailand) equipped with 4-10 MHz phased array cardiac probes (Mindray Medical Co., Ltd., Thailand). After that, the rat was waked up.

**Table 5 Composition of normal diet**

| Composition | Normal diet (CP 082) | | |
|---|---|---|---|
| | G | Kcal | %E |
| Carbohydrate | 495.30 | 1981.20 | 51.99 |
| Fat | 83.70 | 753.30 | 19.77 |
| Protein | 269.00 | 1076.00 | 28.24 |
| Vitamin | 65.40 | - | - |
| Fibre | 34.30 | - | - |
| Total | 947.70 | 3810.50 | 100 |
| Kcal/g | | 4.02 Kcal/g | |

**Table 6 Criteria for diagnosis of diabetic rat (241)**

| Test | Normal range | DM range |
|---|---|---|
| FPG | 80-100 mg/dL | ≥ 126 mg/dL |
| OGTT | < 140 mg/dL | ≥ 200 mg/dL |
| $HbA_{1c}$ | < 5.7 % | ≥ 6.5 % |

**Note:** Abbreviations: FPG; fasting plasma glucose, OGTT; oral glucose tolerance test, $HbA_{1c}$; glycated haemoglobin test

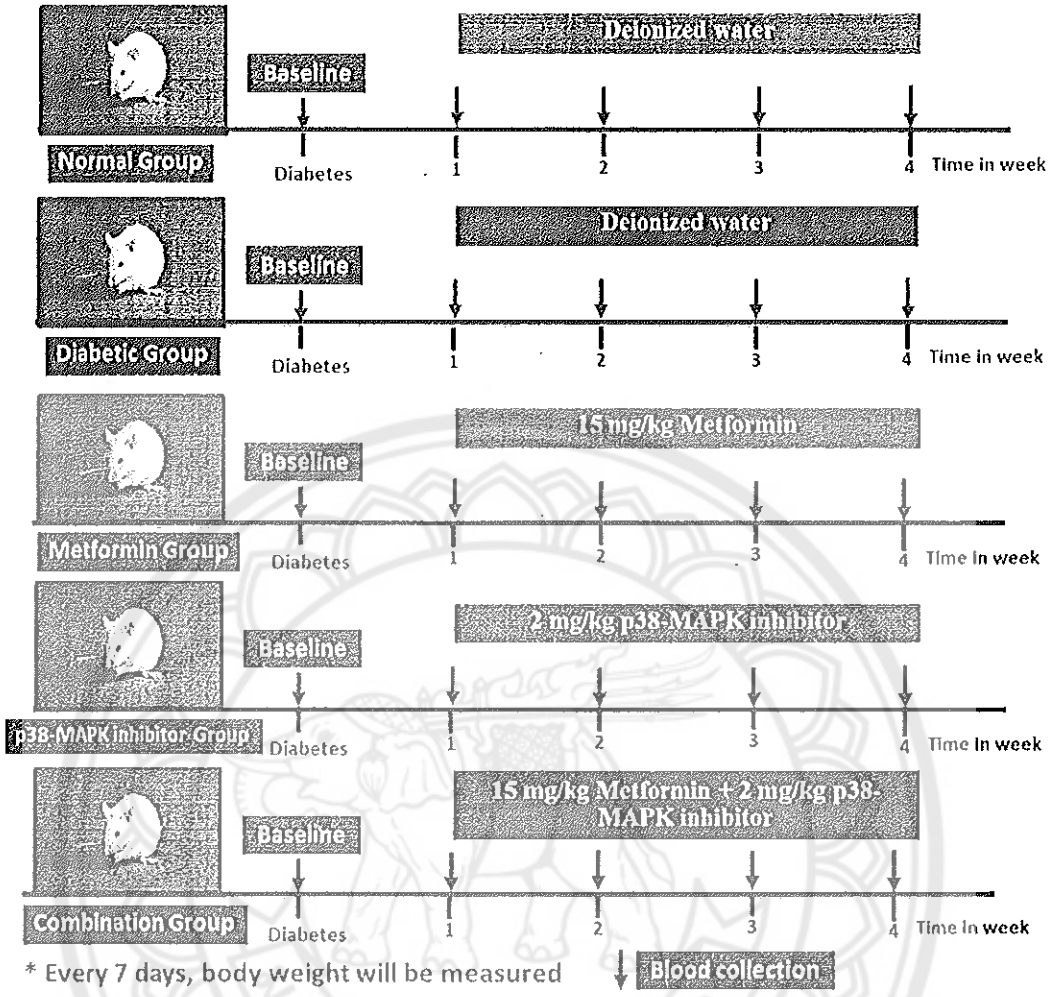


Figure 10 Diagram of experimental design for induced to diabetic condition.

**6. The *ex vivo* myocardial ischemia/reperfusion injury in type 2 diabetic rat by Langendorff perfusion**

6.1 Heart cannulation (Ischemia condition) and reperfusion

The male Wistar rats, as a control group, and GK rats, as a diabetes group and treatment group, were intraperitoneal injected (IP) with pentobarbital (300 mg/kg) and heparin (150 units) for anaesthetic. The heart was rapidly removed and isolated then it was placed in ice cold modified Krebs-Henseleit (K-H) buffer containing 118.5 mM NaCl, 25.0 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4.7H_2O$, 11.0 mM D-glucose and 1.41 mM $CaCl_2.2H_2O$, pH 7.4. The heart was removed the excess thymus and fatty tissue. After that, the aorta was cannulated with 21G blunt and grooved stainless steel needle. The hearts were retrogradely perfused, at the constant pressure of 80 mmHg (108 cm $H_2O$) with K-H buffer (Figure 11). The K-H buffer was maintained a constant temperature at 37°C after filtered by a 0.8 μm micro-filter equilibrated with 95% $O_2$ and 5% $CO_2$ (242). In the stabilization period, heart was perfused with K-H buffer for 30 min. After stabilization, the flow of K-H buffer was closed for 30 min, as an ischemia period. Finally, the flow of K-H buffer was open again in the rat heart for reperfusion for 90 min and the heart was stained with 1% (w/v) TTC dye for measuring the infarct size.

6.2 Exclusion criteria

Predefined exclusion criteria stated that perfused rat hearts were excluded from further study if the baseline values, after 30 min stabilization, do not fulfil the following criteria. (242)

Coronary flow greater than 1 ml/min but less than 5 ml/min

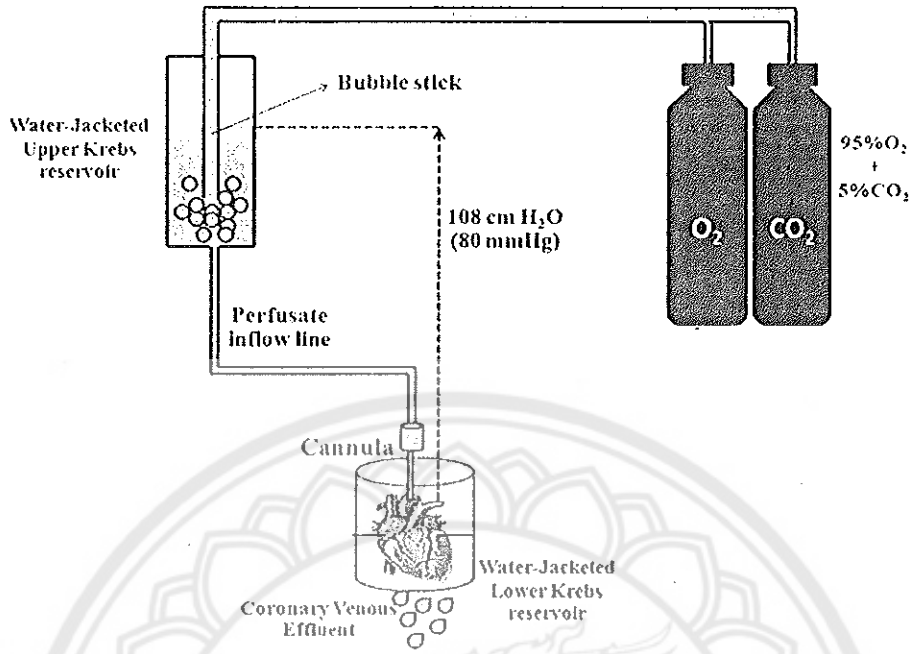


Figure 11 Schematic diagram explaining the Langendorff apparatus used in studies described within this thesis. The heart was perfused by oxygenated K-H buffer maintain at 37°C, at constant pressure 80 mmHg which determine by the high of the upper K-H buffer reservoir. The coronary flow can be indirectly measured as the overflow rate of effluent from the lower reservoir.

6.3 Determination of the effect of metformin combines with p38-MAPK inhibitor (SB203580) on myocardial infarct size during myocardial ischemia in diabetic heart.

The male Wistar rats and the male GK rats, which treated with metformin or SB203580 or metformin + SB203580 (Table 7), were injected with pentobarbital (300 mg/kg) and heparin (150 units) at intraperitoneal (IP) for anesthetize. The heart was perfused for 30 min with K-H buffer containing with metformin or SB203580 or metformin+SB203580 at 37°C after the heart was rapidly excised from the rats. Then, the coronary flow was measured. The hearts were excluded out from this study when after 30 min stabilization the heart does not fulfil the criteria as described above. After 30 min stabilization, the flow of K-H buffer was stopped for 30 min (Ischemia condition). Finally, the K-H buffer was added in the rat heart for reperfusion for 90 min and the heart was stained with 1% (w/v) TTC dye for measuring the infarct size (Figure 12).

**Table 7 The experimental design groups in ex vivo for determination of infarct size**

| Groups | Conditions |
|---|---|
| 1 | Control rats (Wistar rats) |
| 2 | Control diabetic rats (GK rats) |
| 3 | Diabetic + Metformin rats (GK rats) |
| 4 | Diabetic + p38-MAPK inhibitor (SB203580) rats (GK rats) |
| 5 | Diabetic + Metformin + p38-MAPK inhibitor (SB203580) rats (GK rats) |

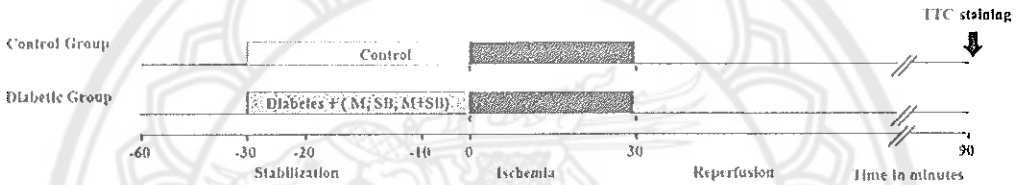


**Figure 12 Schematic diagram explaining the groups of rats for studying the physiologically function of the heart and determining the effect of metformin combine with p38 MAPK inhibitor on the simulated ischemia of diabetes heart.**

6.4 Determination of signal transduction in organ level responding to the combination of between metformin and p38-MAPK inhibitor by Western blotting.

The previous study has been demonstrated that the suitable time for induce p38 MAPK phosphorylation on ischemic condition is 10 min. Therefore, this condition was used to determine the effect of the combination between metformin and SB203580 on signal transduction in organ level responding to ischemic condition (11).

The male Wistar rats and the male GK rats, which treated with Metformin or SB203580 or Metformin + SB203580 (Table 8), were anesthetized and the heart was cannulated. After that, the cannulated heart was perfused with the K-H buffer containing metformin or SB203580 or metformin + SB203580 for 40 min and then, stopped the flow of K-H buffer for 10 min. After 10 min, the hearts were rapidly snapped frozen (Figure 13). The hearts sample were weighed, and the heart was added 500 µl of homogenization buffer for homogenize. The heart sample that homogenates were centrifuged at 14,000 rpm, 4°C for 10 min. Then, the sample was collected and added in equal volume of 2X SDS-PAGE sample buffers; containing 10% (v/v) 2-mercaptoethanol and bromophenol blue dye. The sample was boiled for 10 min. The heart proteins were analysed by Western blotting using specific antibody for phosphorylated-Akt (Santa cruz biotec #sc-7985), total-Akt (Santa cruz biotec #sc-8312), phosphorylated p38 (Santa cruz biotec #sc-17852), total-p38 (Santa cruz biotec #sc-535), Bax (Santa cruz biotec #sc-493), Bcl-2 (Santa cruz biotec #sc-492), and caspase 3 (Santa cruz biotec #sc-7148) (All of primary antibodies were diluted at 1:500 (except p38 was diluted at 1:1,000) in 1% (w/v) skim milk + TBST buffer) and the horseradish peroxidase-conjugated secondary antibody, which diluted at 1:2,000 in 1% (w/v) skim milk + TBST, after separated the heart protein in SDS-PAGE. The band intensity quantitation was determined by Image Lab™ 2.0 Software (BIORAD) (Figure 13).

**Table 8 The experimental design groups in ex vivo for determination of signal transduction in organ level**

| Groups | Conditions |
|---|---|
| 1 | Control rats (Wistar rats) |
| 2 | Control diabetic rats (GK rats) |
| 3 | Diabetic + Metformin rats (GK rats) |
| 4 | Diabetic + p38-MAPK inhibitor (SB203580) rats (GK rats) |
| 5 | Diabetic + Metformin + p38-MAPK inhibitor (SB203580) rats (GK rats) |

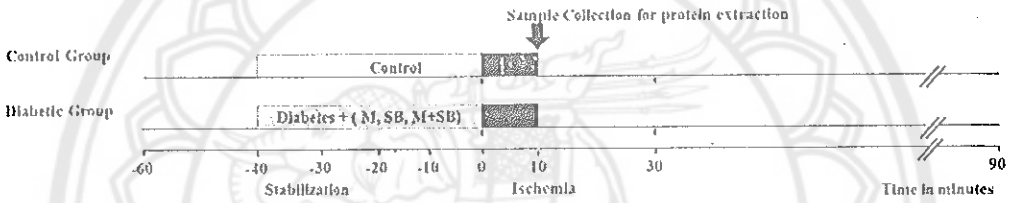


**Figure 13 Schematic diagram explaining the groups of rats for determination of signal transduction in organ level responding to the combination of metformin and p38 MAPK inhibitor.**

6.5 Infarct size measurement

6.5.1 Triphenyl tetrazolium chloride (TTC) staining

1) TTC staining

After reperfusion for 90 min, the heart was incubated with 5 ml of 1% (w/v) triphenyl tetrazolium chloride (TTC) in PBS for 1 min. After incubation, the heart was removed from the cannula and incubated in TTC solution for 10 min at 37°C. Finally, the hearts were removed the atria and were blotted dry and weighted then stored at -20°C for up to 1 week.

2) Sectioning and fixation of heart

Heart was thawed and incubated in 2.5% (v/v) glutaraldehyde for 1 min. Then, the heart was dipped in liquid nitrogen. The dipped hearts were sectioned from apex to base at 1 mm thick slices. After that, the heart slices were incubated in 10% (v/v) formaldehyde overnight at room temperature, then rehydration overnight with PBS at 4°C.

3) Infarct size measurement

The heart sections were putted compress between glass plates (1 mm apart) and scanned at a high solution setting. After that, planimetry was carried out using image analysis software and surface area of the whole, and TTC-negative, myocardium was transformed to volume by multiplication with thickness (1 mm). Within each heart, after summation of individual slices, TTC-negative infarction volume was expressed as a percentage of heart volume. All analyses of infarct size were done by investigator who is blinded with regard to the group assignments.

**Statistical analysis**

All values were expressed as mean ± SD. All comparisons involving more than one group was assessed for significance using one-way analysis of variance (ANOVA), followed when appropriate by the Tukey-Kramer test. Some parameters, which were compared between more than one group or at different time points, was assessed for significance using two-way analysis of variance (ANOVA). A $p$-value less than 0.05 was considered as statistical significance.

# CHAPTER IV

# RESULTS AND DISCUSSION

p38 MAPK inhibitor or SB203580 is one of selective inhibitor known to inhibit p38-MAPK activity by competitively binding the adenosine triphosphate (ATP) site at p38-MAPK ATP binding site, which then inhibit the activity of p38 MAPK to phosphorylate its downstream substrates (146). It is widely used in many studies including myocardial I/R, which could significantly reduce cell death (*in vitro*), infarct size, and improved cardiac function (6, 215, 243).

Metformin is anti-diabetic drugs. It is recommended as the first line drug to control glycaemic status in diabetic patients. Metformin maintain glucose level in plasma by stabilizing and suppressing hepatic glucose production (108). There is evidence suggested that metformin provide cardioprotection against myocardial I/R injury (21).

Both of SB203580 and metformin could show properties of cardioprotective effect on I/R injury. However, the cardioprotective effect of SB203580 in combination with metformin on the diabetic heart subjected to ischemia/reperfusion injury, which the major cause of mortality in diabetic patient, have not been investigated. In this thesis, we determined the effect of combination between SB203580 and metformin on diabetic model, both an *in vitro* and an *in vivo* treatment, followed by myocardial ischemia/reperfusion injury. This study the H9c2 cell line was induced to hyperglycaemia subjected to simulated ischemia/reperfusion (sI/R) condition. Then, the effect of anti-diabetic drug (Metformin) and p38 MAPK inhibitor (SB203580), which treated on hyperglycaemia subjected to ischemia/reperfusion injury condition, was observed.

**Determination of metformin and SB203580 in hyperglycaemic condition.**

**1. Optimization of hyperglycaemic condition on cardiac cell line.**

An *in vitro* hyperglycaemic condition was performed by the incubating of H9c2 cell in culture medium supplemented with high concentration of D-glucose. In this study, we optimized the concentration of D-glucose to induce hyperglycaemic condition in H9c2 cell line. Then, the viability of H9c2 cell was determined. The results showed that incubation with glucose solution at the concentrations is 11 mM, 22 mM, 33 mM and 60 mM for 24 h did not induced cell death in H9c2 cell when compared to control group (100.00 ± 0.00 % vs 98.16 ± 11.10 %, 102.2 ± 10.39 %, 100.8 ± 10.00 %, 99.75 ± 12.61 %, respectively). Similar to the incubation at 48 h (100.00 ± 0.00 % vs 98.44 ± 8.19 %, 99.37 ± 10.66 %, 102.7 ± 10.56 %, 102.1 ± 8.77 %, respectively), at 72 h (100.00 ± 0.00 % vs 96.75 ± 8.43 %, 99.91 ± 12.51 %, 97.83 ± 13.96 %, 100.4 ± 9.428 %, respectively) and at 96 h (100.00 ± 0.00 % vs 94.77 ± 17.34 %, 97.57 ± 14.48 %, 95.89 ± 16.35 %, 100.1 ± 17.26 %, respectively), the glucose solution did not induce H9c2 cell death when compared to control group.

To avoid the effect of osmolality induced cell toxicity, the viability of H9c2 cell after culture medium supplemented with similar concentration of mannitol was performed. The results showed that incubation with mannitol solution at the concentrations is 11 mM, 22 mM, 33 mM for 24 h the H9c2 cell viability did not reduced whereas in 60 mM significantly reduced H9c2 cell viability when compared to control group (100 ± 0.00 % vs 102.9 ± 10.03 %, 101.8 ± 8.77 %, 101.7 ± 10.76 %, 93.40 ± 7.91 %, respectively; $p<0.05$). For 48 h of incubation, all of concentration (11 mM, 22 mM, 33 mM and 60 mM) did not decreased cell viability of H9c2 cell when compared to control group (100 ± 0.00 % vs 98.03 ± 7.17 %, 96.28 ± 11.40 %, 95.00 ± 12.68 %, 93.13 ± 8.02 %, respectively). For 72 h incubation, at the 11 mM and 22 mM of mannitol solution did not decrease H9c2 cell viability (100 ± 0.00 % vs 94.72 ± 4.83 %, 93.63 ± 4.86 %, respectively), in contrast to 33 mM and 60 mM concentration significantly reduced in H9c2 cell viability (100 ± 0.00 % vs 91.54 ± 6.83 %, 87.28 ± 6.90 %, respectively; $p<0.05$), when compared to control group. In addition, incubation with mannitol solution at 11 mM, 22 mM and 33 mM for 96 h did not induced H9c2 cell death (100 ± 0.00 % vs 95.25 ± 7.12 %, 98.88 ± 6.29 %, 95.32 ± 5.48 %, respectively),

whereas at 60 mM could induce significantly H9c2 cell death (100.00 ± 0.00 % vs 91.07 ± 5.44 %, $p$ <0.05). (Figure 14-15).

In this study, we used various concentration of D-glucose solution for mimic hyperglycaemic condition in H9c2 cell, but this method failed to induce toxicity in H9c2 cell. Therefore, we used the concentration and time of glucose solution for inducing hyperglycaemic condition in H9c2 cell line is 33 mM (594 mg/DL) for 24 h, which was the concentration reported in several previous study and this concentration is more than the level of glucose level that used diagnosis diabetic disease in human according to the American Diabetes Association (ADA) recommendation (241).

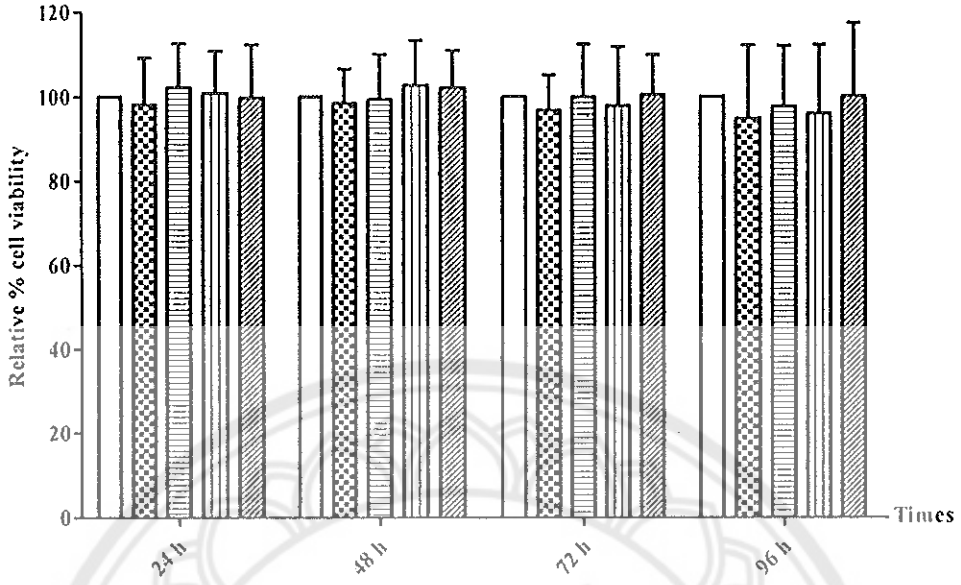


**Figure 14 Determination of hyperglycaemic condition (D-glucose) on cardiac cell line. H9c2 cells were incubated in D-glucose solution at 5.5 mM (Control), 11 mM, 22 mM, 33 mM and 60 mM for 24 h, 48 h, 72 h and 96 h. After incubation, the read-out measurement was MTT cell survival assay. Each bar graph represents means ± SD (n=6).**

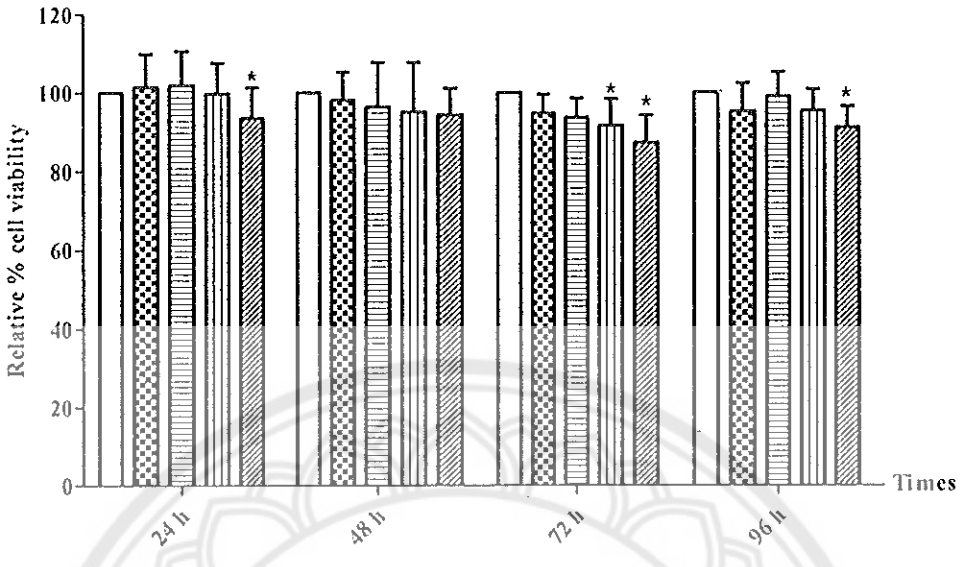


Figure 15 Determination of osmolality control by mannitol solution on cardiac cell line. H9c2 cells were incubated in mannitol solution at 5.5 mM (Control), 11 mM, 22 mM, 33 mM and 60 mM for 24 h, 48 h, 72 h and 96 h. After incubation, the read-out measurement was MTT cell survival assay. Each bar graph represents means SD (n=6). $*p < 0.05$ vs control.

**2. The effect of metformin on hyperglycaemic condition.**

In this experiment, we hypothesized that metformin could increase the viability of H9c2 cell in hyperglycaemic condition. The H9c2 cell was treated with or without 3 mM of metformin for 24 h, to represent the effect of metformin on normoglycemic condition. In hyperglycaemic condition, cells were incubated with culture medium supplemented with 33 mM D-glucose in 3 mM of metformin for 24 h. After that, the viability of H9c2 cell was determined by MTT assay. The results showed that treatment with 3 mM metformin in hyperglycaemic condition was increased in cell viability, but not significantly when compared to control and hyperglycaemic condition without treatment of metformin (109.0 ± 15.80 % vs 100.00 ± 0.00 % and 99.36 ± 12.83) (Figure 16).

**3. The effect of SB203580 on hyperglycaemic condition.**

In this experiment, we proved the effect of SB203580 on H9c2 cell viability, particularly in hyperglycaemic condition. The H9c2 cell was treated with 10 μM of SB203580 for 24 h, to represent the effect of SB203580 on normoglycemic condition. In hyperglycaemic condition, cells were incubated with culture medium supplemented with 33 mM D-glucose in 10 μM of SB203580 for 24 h. The results showed that treatment with SB203580 in hyperglycaemic condition was tended to increase the viability of H9c2 cell but not significant when compared to hyperglycemic condition and control condition (106.6 ± 6.72 % vs 99.36 ± 12.83 % and 100.00 ± 0.00 %) (Figure 16).

**4. The effect of metformin and SB203580 on hyperglycaemic condition.**

The H9c2 cell was induced hyperglycaemic condition and hyperglycaemic condition treated with 3 mM of metformin combined with 10 μM of SB203580 for 24 h. After that, the viability of H9c2 cell was determined by MTT assay. The results showed that the combination between metformin and SB203580 was tended to decrease in cell viability of H9c2 cell (93.88 ± 8.52 % vs 99.36 ± 12.83 %), which induced hyperglycaemic condition. However, treatment with combine drugs did not induced apoptosis in hyperglycaemic condition and this concentration could use in next experiment (Figure 16).

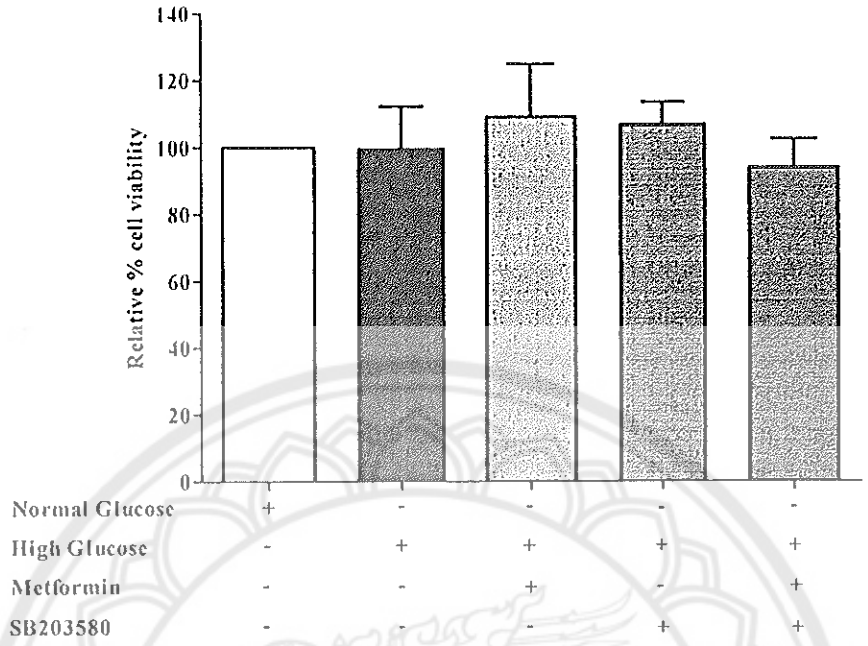


Figure 16 Determination of hyperglycaemic condition treatment with metformin or SB203580 or the combination between metformin and SB203580 on H9c2 cell. H9c2 cells were induced hyperglycaemic condition by using 33 mM of D-glucose solution for 24 h. Then, the induced hyperglycaemic cells were treated with 3 mM of metformin or 10 μM of SB203580 or 3 mM of metformin combine to 10 μM of SB203580 for 24 h. After 24 h of treatment, the read-out measurement was MTT cell survival assay. Each bar graph represents means ± SD (n=6).

**Determination effect of combination between metformin and SB203580 on cellular reactive oxygen species (ROS) level in hyperglycaemic condition.**

Previous studies have been reported that the increasing of intracellular ROS production leading to cell injury and cell death in hyperglycaemic or diabetic condition. ROS induces the cellular damage through many pathways including activation of pro-apoptotic pathways, and induces of the inflammatory in cellular (244-246). Since, ROS play a major role on hyperglycaemic condition, and metformin and SB203580 that acts as the artificial anti-oxidant. Therefore, the capability of high-glucose induced intracellular ROS production and the cardioprotective effect of drugs treatment in hyperglycaemia was investigated. In this experiment, the H9c2 cell was treated with drugs and carboxy-$H_2$DCFDA dye for 24 h. Then, the cellular ROS level was measured. The results demonstrated that hyperglycaemic condition (33 mM D-glucose) was significantly induced intracellular ROS production (2832 ± 289.3 A.U.) when compared to control (2305 ± 312.3 A.U.) ($p$<0.05, ANOVA). Treatment with SB203580 in hyperglycaemic condition was significantly decreased ROS level when compare to hyperglycaemic group (2832 ± 289.3 A.U. vs 2399 ± 188.0 A.U., $p$<0.05; ANOVA). Moreover, treatment with metformin (2598 ± 125.3 A.U.) or combine drugs (2615 ± 237.4 A.U.) in hyperglycaemic condition no significant difference reduces in ROS production when compared to hyperglycaemic condition. These results indicated that induction hyperglycaemic in cardiac cell line could increase the ROS production and the ROS product, which induced by hyperglycaemic condition, could reduce by SB203580 treatment. Therefore, in this study the SB203580 showed the improvement on cellular reactive oxygen species (ROS) level in hyperglycaemic condition (Figure 17).

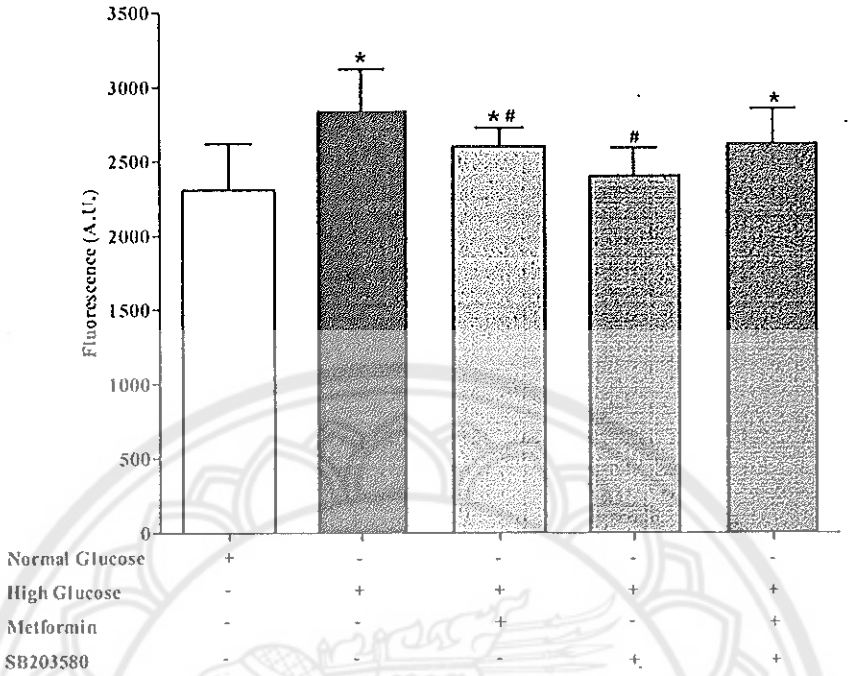


Figure 17 Determination of cellular reactive oxygen species (ROS) level in hyperglycaemic condition after treatment with metformin or SB203580 or the combination between metformin and SB203580. The H9c2 cell was incubated with carboxy-$H_2$DCFDA and high-glucose solution that treated with metformin or SB203580 or combine drugs for 24 h. After that, the ROS production was assessed by fluorescence detector. Each bar graph represents means ± SD (n=6). $^{*}p < 0.05$ vs control and $^{\#}p < 0.05$ vs high-glucose.

**Determination of metformin and SB203580 in hyperglycaemia subjected to simulated ischemia/reperfusion injury (sI/R) condition.**

**1. Optimization of simulated ischemia/reperfusion injury condition.**

H9c2 cells were induced simulated ischemia for 20, 40, 60, 120 and 180 min and reperfusion for 24 h. After 24 h of reperfusion, the cells were measured viability by MTT assay. The results showed that simulated ischemic/reperfusion at 20, 40, 60, 120 and 180 min could significantly reduce cell viability when compared to control (100.00 ± 0.00 % vs 68.70 ± 6.22 %, 56.78 ± 5.31 %, 27.54 ± 6.90 %,, 1.007 ± 0.83 % and 0.53 ± 0.01 %, respectively; $p<0.05$) (Figure 18). The optimal condition that gave 50% cell viability of H9c2 cell after incubation was 40 min. Therefore, the sI incubation time at 40 min and reperfusion at 24 h was an optimal time and it was used in all sI/R experiments for determination of cell viability.

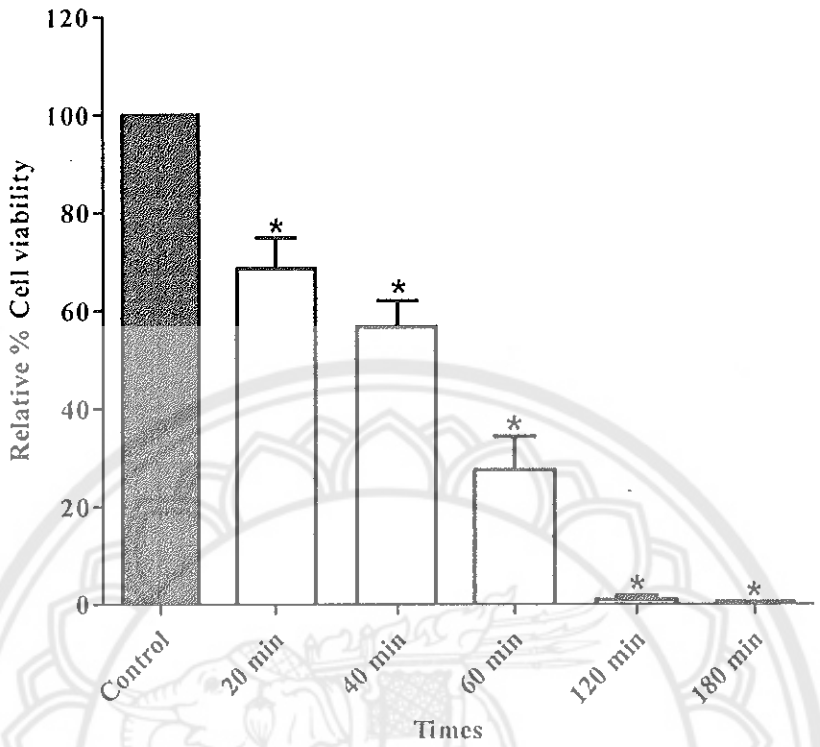


Figure 18 Optimization of simulated ischemia/reperfusion (sI/R) duration. The H9c2 cells were incubated with sI buffer at various periods, including 20 min, 40 min, 60 min, 120 min, and 180 min. the read-out measurement was MTT cell survival assay. Each bar graph represents means ± SD (n=6). for each the 6 experiments. * $p < 0.05$ vs control group.

**2. The effect of simulated ischemia/reperfusion injury on hyperglycaemia.**

After the optimal condition of sI/R was determined. Then, the effect of hyperglycaemia subjected to sI/R condition on cardiac cell death was investigated. The H9c2 cell was induced to hyperglycaemic condition for 24 h. Then, the simulated ischemia was performed for 40 min followed by reperfusion for 24 h. After 24 h of reperfusion, the cell viability was determined. The results showed that the cell viability of H9c2 cell was significantly reduced after induced sI/R condition or induced hyperglycaemia subjected to sI/R condition when compared to control condition (100.00 ± 0.00 % vs 81.50 ± 7.52 %, 59.92 ± 16.51 %, respectively; $p<0.05$). In addition, after induced hyperglycaemia subjected to sI/R the H9c2 cell was significantly decreased cell viability from 81.50 ± 7.52 % to 59.92 ± 16.51 %, $p<0.05$ when compared to sI/R condition alone (Figure 19). This result indicated that the hyperglycaemia subjected to simulated ischemia/reperfusion injury condition could increase cardiac cell death more than hyperglycaemic condition alone.

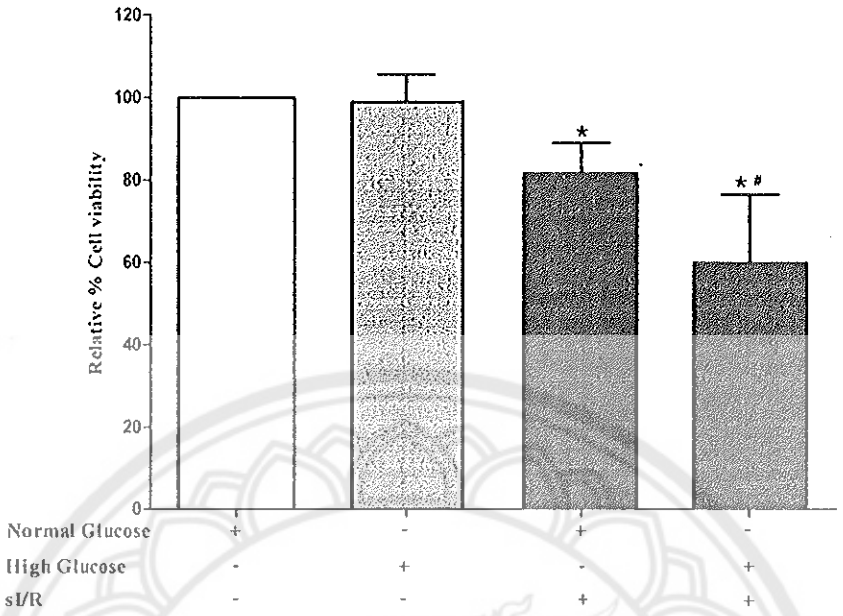


Figure 19 Determination of hyperglycaemia subjected to simulated ischemia/reperfusion (sI/R) on H9c2 cell. H9c2 cells were incubated in D-glucose solution at 33 mM for 24 h. Then, the induced cells were simulated ischemia/reperfusion (sI/R). After that, the read-out measurement was MTT cell survival assay. Each bar graph represents means ± SD (n=6). $^{*}p < 0.05$ vs control and $^{\#}p < 0.05$ vs sI/R.

**3. The effect of metformin on hyperglycaemia subjected to simulated ischemia/reperfusion injury.**

Metformin is an anti-diabetic drug, which widely used for diabetic treatment. Moreover, it has been evidence indicated that metformin show cardioprotective *in vitro* studies (21). From this information leading to our hypothesized that metformin could improve cardiac cell death in hyperglycaemia subjected to ischemia/reperfusion injury condition.

Therefore, in this experiment the H9c2 cell was induced in hyperglycaemia in the presence of 3 mM of metformin for 24 h. Then, the cells were subjected to simulated ischemia for 40 min and reperfusion for 24 h. After 24 h of reperfusion, the cells were measured viability by MTT assay. The results showed that the relative of death cell in hyperglycaemia subjected to sI/R or hyperglycaemia subjected to sI/R treated with metformin were significantly increased when compare to sI/R in normal condition ($1.00 \pm 0.00$ vs $1.61 \pm 0.09$ or $1.43 \pm 0.17$, $p<0.05$; ANOVA). However, treatment with metformin could significantly reduce cell death when compared to hyperglycaemia with sI/R injury ($1.61 \pm 0.09$ vs $1.43 \pm 0.17$, $p<0.05$; ANOVA). (Figure 20). From this result could indicate that metformin showed cardioprotective effect by improving cardiac cell death in hyperglycaemia subjected to sI/R condition. Therefore, this concentration was used in next experiment for determination of cell viability.

**4. The effect of SB203580 on hyperglycaemia subjected to simulated ischemia/reperfusion injury.**

The H9c2 cell was induced hyperglycaemic condition in the presence and absence of 10 μM of SB203580 for 24 h. Then, all group of cells were subjected to simulated ischemia for 40 min and reperfusion for 24 h. After 24 h of reperfusion, the cells were measured viability by MTT assay. The results showed that cell death in both of hyperglycaemia subjected to sI/R condition or hyperglycaemia subjected to sI/R treated with SB203580 condition were significantly increased when compare to sI/R group ($1.00 \pm 0.00$ vs $1.61 \pm 0.09$ or $1.39 \pm 0.10$, $p<0.05$; ANOVA). In addition, the cell death was significantly decreased from $1.61 \pm 0.09$ to $1.39 \pm 0.10$ ($p<0.05$; ANOVA) after treated with 10 μM of SB203580 in hyperglycaemia subjected to sI/R condition when compare to hyperglycaemia subjected to sI/R condition alone (Figure 20). This result demonstrated that treatment with SB203580 showed cardioprotective effect on

cardiac hyperglycaemia subjected to sI/R condition by increasing cell viability in hyperglycaemia subjected to sI/R condition. This concentration was used in next experiment for determination of cell viability.

**5. The effect of combination of metformin and SB203580 on hyperglycaemia subjected to simulated ischemia/reperfusion injury.**

The next experiment we hypothesized that the combination between metformin and SB203580 could reduce cardiac cell death in hyperglycaemia subjected to sI/R injury.

H9c2 cell was induced hyperglycaemic condition (33 mM of D-glucose solution) or hyperglycaemia treatment with 3 mM of metformin combine with 10 μM of SB203580 for 24 h. Then, the sI/R condition was performed. Finally, the cells were measured viability by MTT assay. The results demonstrated that the number folds of death cell in all groups subjected to sI/R with or without treatment groups were significantly increased when compared to sI/R group (1.00 ± 0.00 vs 1.61 ± 0.09 (sI/R + High-Glucose (HG)), 1.43 ± 0.17 (sI/R + HG + Met), 1.39 ± 0.10 (sI/R + HG + SB) and 1.48 ± 0.06 (sI/R + HG + Met + SB), $p<0.05$; ANOVA) (Figure 20). Moreover, the cell death was significantly decreased in hyperglycaemia subjected to sI/R treatment with metformin, (1.43± 0.17) or SB203580 (1.39 ± 0.10) or combination between metformin and SB203580 group (1.48 ± 0.06), when compared to hyperglycaemia subjected to sI/R group. From these results, demonstrated that treatment with the combination between metformin and SB203580 showed cardioprotective effect on cardiac hyperglycaemia subjected to sI/R condition (Figure 20).

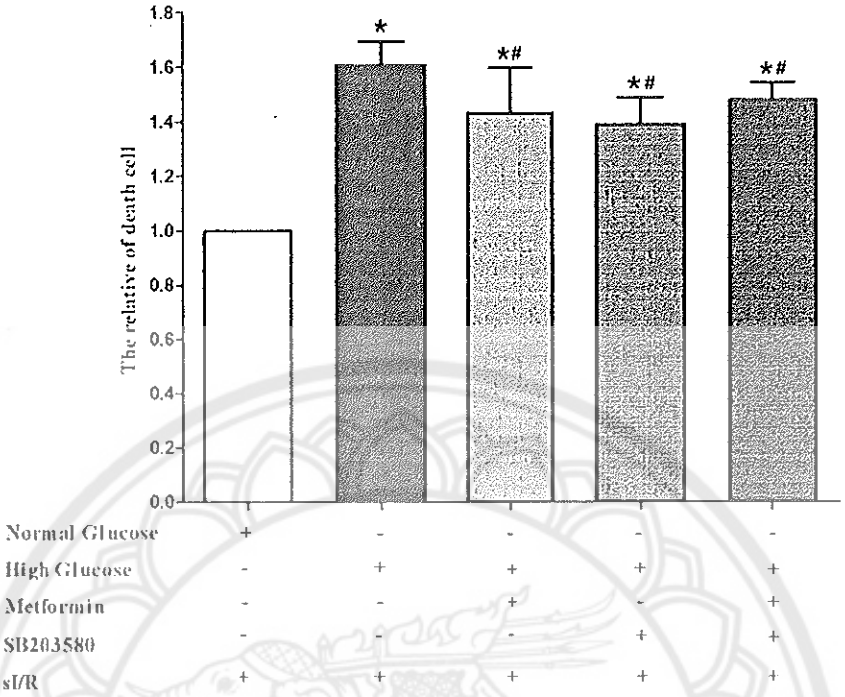


Figure 20 Determination of the effect of metformin or SB203580 or the combination between metformin and SB203580 in hyperglycaemia subjected to sI/R injury. The H9c2 cells were induced hyperglycaemia and treated with or without 3 mM of metformin or 10 μM of SB203580 or combination between metformin and SB203580 for 24 h. Then, the cells were induced sI/R condition. After that, the read-out measurement was MTT cell survival assay. Each bar graph represents means SD (n=6). $^{*}p < 0.05$ vs sI/R and $^{\#}p < 0.05$ vs sI/R + High-Glucose.

**Determination of metformin and SB203580 in hyperglycaemia subjected to simulated ischemia/reperfusion injury (sI/R) condition on signal transduction in cellular level.**

In this study, we hypothesized that the combination drugs reduced the worsening in cardiac cell on hyperglycaemia subjected to sI/R condition by inhibition of p38 MAPK activation and increasing of Akt activation that plays a role in the survival pathway of cells. The H9c2 cell were treated with or without drugs and then induced hyperglycaemia subjected to sI/R condition. After that, the cellular protein was collected and the cellular signalling was determined by Western blot analysis.

The result show that the hyperglycaemic condition and hyperglycaemia subjected to sI/R condition were significantly increased p38 MAPK phosphorylation when compared to control (1.1 ± 0.40 and 0.808 ± 0.33 vs 0.41 ± 0.23, $p<0.05$; ANOVA, Figure 21). Treatment with metformin or SB203580 or combination between metformin and SB203580 was significantly inhibit p38 MAPK activation when compared to hyperglycaemia subjected to sI/R condition (0.103 ± 0.05 or 0.27 ± 0.11 or 0.181 ± 0.07 vs 0.808 ± 0.33, $p<0.05$; ANOVA, Figure 21). Moreover, we also determined the phosphorylation of Akt, the result showed that the hyperglycaemic condition and hyperglycaemia subjected to sI/R condition reduced Akt phosphorylation, but not significantly different when compared to control group (0.83 ± 0.36 and 0.46 ± 0.20 vs 1.06 ± 0.39, $p<0.05$; ANOVA, Figure 22). Treatment with metformin or SB203580 or combination between metformin and SB203580 could not significantly enhance Akt phosphorylation when compared to hyperglycaemia subjected to sI/R condition (0.50 ± 0.20 or 0.54 ± 0.12 or 0.59 ± 0.15 vs 0.46 ± 0.20, $p<0.05$; ANOVA, Figure 22). These results indicated that hyperglycaemic condition and hyperglycaemia subjected to sI/R condition could induce cardiac cell death by phosphorylation of p38 MAPK and reduced Akt phosphorylation. Treatment with metformin or SB203580 or combination between metformin and SB203580 could improve cardiac cell death by inhibition of p38 MAPK phosphorylation but not sufficient to enhance Akt phosphorylation.

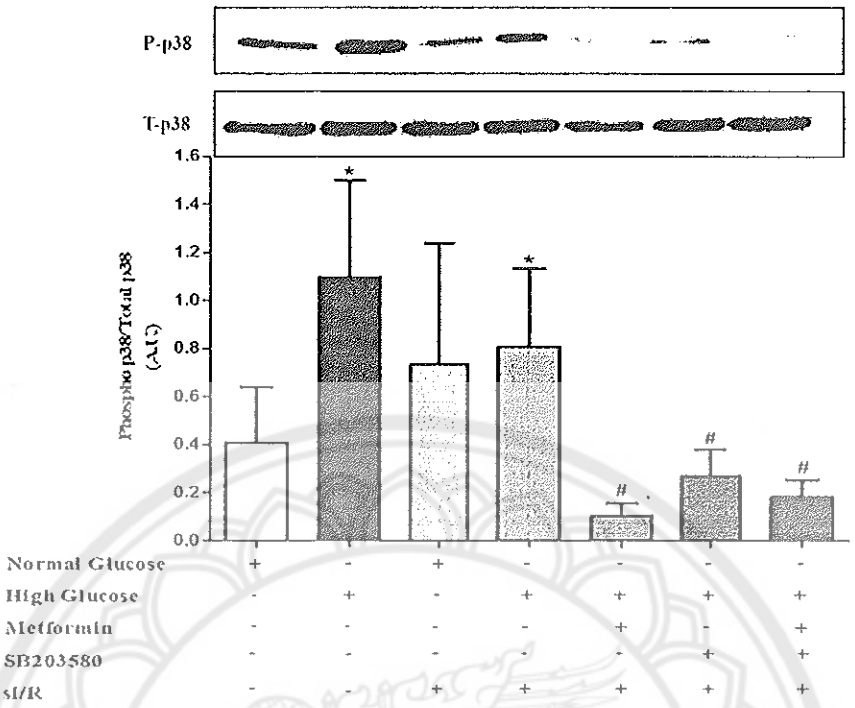


Figure 21 Determination of p38 MAPK phosphorylation in H9c2 cell line hyperglycaemia subjected with sI/R. The cellular protein was assessed p38 MAPK phosphorylation, after induced hyperglycaemia subjected with sI/R condition, by using Western blotting analysis. Each bar graph represents means SD (n=6). $*p < 0.05$ vs control, $^{\#}p < 0.05$ vs sI + HG.

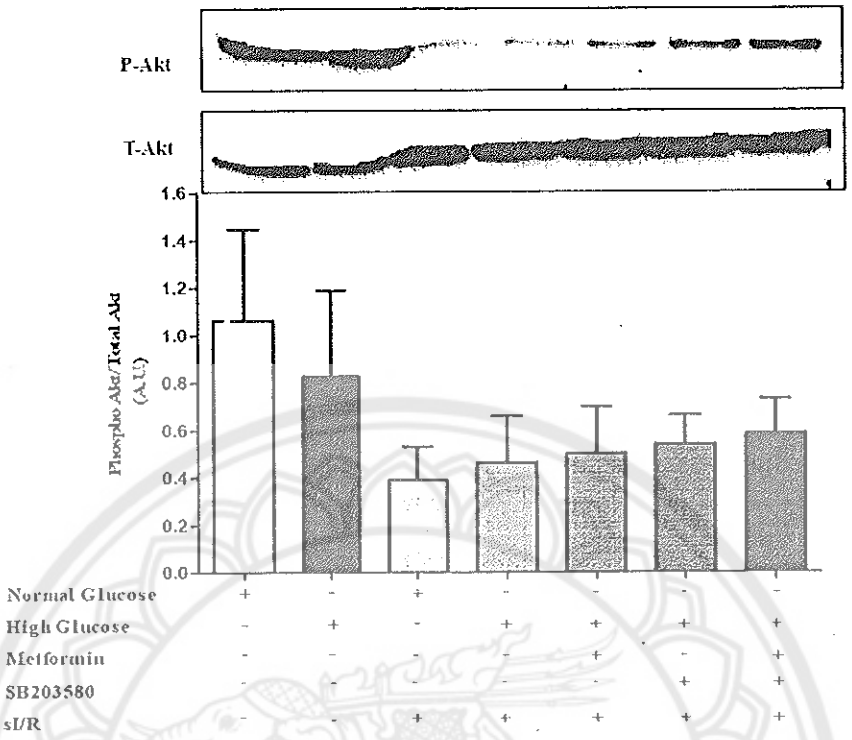


**Figure 22 Determination of Akt phosphorylation in H9c2 cell line hyperglycaemia subjected with sI/R. The cellular protein was determined Akt phosphorylation, after induced hyperglycaemia subjected with sI/R condition, by using Western blotting analysis. Each bar graph represents means SD (n=6).**

Type 2 diabetes is a major cause of mortality and morbidity. It was predicted to increase in every year (1). From this problem leading to several researches attempt to mimic hyperglycaemic or diabetic condition *in vitro,* for develop a new diabetic treatment that could decrease risk of diabetes prevalence. Most of hyperglycaemic studies have been used D-glucose solution for stimulates hyperglycaemic condition. In 2013, Wenming Xu et. al. used 35 mM of glucose at 24 h for inducing hyperglycaemic condition to H9c2 cell. In this study they found that after incubation the viability of H9c2 cell was decreased when compare to control (Normal condition) (247). Similar to another hyperglycaemic studies that used various concentration of high-glucose solution for inducing hyperglycaemic condition including 25 mM, 30 mM, 33 mM and 33.3 mM (229, 248-250). Most of these studies, they found that high glucose has a toxic and decreasing to cell viability. In contrast to our study, we used various concentration of D-glucose solution for mimic hyperglycaemic condition in H9c2 cell, but this method failed to induce toxicity in H9c2 cell. This finding similar to the study of Gunter Wolf and colleagues in 1992, they were reported that the high-glucose concentration could enhance murine mesangial cells (MMCs) proliferation at the early phase (24 h and 48 h) was associated with induction of the S-phase related proliferating nuclear cell antigen (PCNA) as well as the increase expression of the immediate early genes *c-myc* and *egr-1* (251). The previously study has been demonstrated that the incubation with high-glucose concentration could induce cell proliferation of H9c2 cell and HeLa cell, which was associated with Gene Associated with Retinoid-IFN-Induced Mortality 19/STAT3 (GRIM-19/STAT3) signalling pathway (252). Moreover, it has been reported that the H9c2 cell proliferation, which induce by glucose and insulin, could regulate through influencing glucose transporter 4 (GLUT4) expression (253). From these evidences, the one of reason to enhance H9c2 cell proliferation by high-glucose stimulation may be associated with the effect of induction in GRIM-19/STAT3 signalling pathway or the regulation of GLUT4 expression. Therefore, the stimulation of the GRIM-19/STAT3 signalling pathway or GLUT4 expression in high-glucose concentration need to be further investigated.

From the non-toxicity in high-glucose problem, we were looked for other ways to determination the toxicity of high-glucose in cardiac cells line.

The reactive oxygen species (ROS) is one of intracellular production through multiple mechanisms, which plays a major role on hyperglycaemia induced cell death and cell injury during diabetic condition. The increasing of ROS level in hyperglycaemic or diabetic condition could induce the cellular damage and cellular apoptosis including induced apoptosis by activation of pro-apoptotic pathways (244), and recruitment of the inflammatory cells (245). In 2010, Dachun Yao and Michael Brownlee reported that the ROS production, which induced by hyperglycaemic condition, cause of diabetic tissue damage and chronic inflammation by increasing expression of RAGE and RAGE ligands (254). Moreover, there was evidence demonstrated that incubation of high-glucose solution in endothelial cells could induce apoptotic cell death by ROS elevation (246). In addition, the accumulation of ROS in hyperglycaemic condition could promote the diabetic complication including diabetic retinopathy and diabetic cardiomyopathy (245). In our study, the determination of high-glucose induced intracellular ROS production was performed for assessment the toxicity of high-glucose in cardiac cells line. Our findings showed that incubation of high-glucose solution in H9c2 cell increased intracellular ROS production in cardiac cells. Therefore, as previously mentioned, the high-glucose solution causes of toxicity in cardiac cell by increasing the intracellular ROS production.

The progression of diabetes leading to develop of several diabetic complications such as retinopathy, nephropathy, neuropathy, peripheral vascular disease and coronary heart disease (4, 37). Especially cardiovascular diseases, it has been reported that diabetic patients have a higher risk of cardiovascular diseases around two to four times than non-diabetic (5). In 2016, Mingyan Hu et. al. induced hyperglycaemia in H9c2 cell by using 33 mM of glucose solution then subjected to hypoxia/reoxygenation (H/R) injury. They found that cell viability was decrease after induced hyperglycaemia subjected to H/R condition (255). In addition, it has been reported that hyperglycaemia subjected to ischemia/reperfusion could aggravate apoptosis in H9c2 cell (256, 257). Similar to our experiment, we found that hyperglycaemia subjected to ischemia/reperfusion injury could reduce H9c2 cell viability. From this result suggested that ischemia/reperfusion injury condition is a cause of worsening on hyperglycaemic cardiac cell.

Metformin is a one of anti-diabetic drug that was recommended to the first line drug for treatment in diabetic patient. Metformin could enhance the action of insulin in the liver to decrease the glucose product of hepatic and reduce glucose level in hyperglycaemic condition (19, 258). The previous study reported that metformin could improve hyperglycaemia-induced cell apoptosis through many pathways including enhancing autophagy activity and restored Cx43 expression in hyperglycaemia in cardiac cell line (259), prevented high glucose-induced premature senescence in endothelial cells (260) and effect through inhibition of ceramide synthesis in high-fat-induced cardiac cell death (261). In addition, there was previously evidence reported that metformin showed cardioprotective effect in ischemia/reperfusion injury condition (21) and hyperglycaemia subjected to Hypoxia/Reoxygenation (H/R) Injury condition (255). From this information, we hypothesized that metformin could improve cell death in hyperglycaemic condition and hyperglycaemia subjected to ischemia/reperfusion injury. Our hyperglycaemic study, we found that high-glucose condition induced H9c2 cell proliferation rather than induced H9c2 cell death, however, treatment of metformin in H9c2 cell, which induced in high-glucose condition, also significantly increased H9c2 cell proliferation when compare to induce high-glucose condition alone. This finding indicated that the increasing of H9c2 cell viability in hyperglycaemic condition after treated with metformin could be considered as an effective treatment of H9c2 cells in hyperglycaemic condition. From this situation, metformin increased H9c2 cell proliferation in high-glucose condition, may be owing to the association with the effective of metformin to activate the GRIM-19/STAT3 signalling pathway in H9c2 cell induce high-glucose condition. The previous evidence has been demonstrated that the activation of the GRIM-19/STAT3 signalling pathway in H9c2 cell was associated with cell proliferation and the effect of metformin induce the GRIM-19/STAT3 signalling pathway in high-glucose condition was remains unknown (252). Therefore, the accurate of metformin induce the GRIM-19/STAT3 signalling pathway in high-glucose was investigated to elucidate the effective of metformin to improve high-glucose induced H9c2 cell injury. Since in our findings, we found difference results of metformin treated H9c2 cell in high-glucose condition, one result was increased H9c2 cell proliferation and another result did not further effect in H9c2 cell proliferation. However, the direct effect of metformin treatment in hyperglycaemic condition was clearly seen, if high-

glucose solution reduced cell viability of H9c2 cell. Moreover, the improvement of cardiac cell death in hyperglycaemia subjected to sI/R condition by metformin was also demonstrated in this study. Therefore, these findings suggested that metformin could improve cardiac cell on hyperglycaemic condition and hyperglycaemia subjected to sI/R condition.

The previously study has been demonstrated that treatment with metformin in rat glomerular mesangial cells (MCs) could reduce ROS production in high-glucose condition by increasing the activity of SOD (262). Moreover, the effective of metformin have been reported that the metformin could improve mitochondrial derived ROS (mtROS) hyperglycaemia-induced cell death in permeabilized human microvascular endothelial cells (HMEC-1) by prevention of the mitochondrial permeability transition pore (PTP) opening–related cell death (263) and in Human Umbilical Vein Endothelial Cells (HUVECs) through AMPK–PGC-1α–related mechanism (264, 265). Our finding showed treatment with metformin in H9c2 cell induced high-glucose condition could non-significantly reduce ROS production. In our finding showed the less effective of metformin to reducing high-glucose induce ROS production may be due to dose of metformin that used in treatment and type of cell because in previously studies were used in various dose of metformin and study in many cell types. Therefore, the optimization of metformin dose in high-glucose induce ROS generation was further determined.

The p38 MAPK is one of serine/threonine protein kinases that act as cellular responses to external stress signalling and several cellular processes such as cell differentiation, inflammation, cell proliferation and cells death (125, 126). It has been reported that p38 MAPK could activate by hyperglycaemic condition (266). The appropriate way for inhibit p38 MAPK activation is used p38 MAPK inhibitor. The SB203580 (p38 MAPK inhibitor), which widely used in many diabetic experiments. The previous evidence has been reported that the concentration of SB203580 at 10 μM was frequently used to establish the involvement of p38-MAPK in cardiac cellular responses that completely inhibits at least one of kinase in the heart (54 kDa SAPK/JNK) (8). Moreover, it has been reported that SB203580 could improve cell death in hyperglycaemic condition by decreasing the expression levels of cleaved caspase3/caspase3 and Bax/$Bcl_2$, which is cellular apoptotic pathway (267, 268). In

contrast to our study, we found that treatment with SB203580 in high glucose condition did not affect to cell viability of H9c2 cell. From this result indicated that SB203580 has not affect in normal condition and hyperglycaemic condition, this may be due to high glucose solution did not induced cell death on H9c2 cell. However, treatment with SB203580 did not cause of toxicity in H9c2 cell. In addition, treatment of SB203580 could improve cardiac cell death (6) in ischemia/reperfusion condition and it was showed cardioprotective effect, including improved left ventricular function, reduced cardiac inflammation and improve cardiac contractile functions on insulin resistance or diabetic models (21). However, the effect of SB203580 on ischemia/reperfusion in cardiac hyperglycaemia have not been investigated. Therefore, in this study we were determined the effect of SB203580 on cardiac hyperglycaemia subjected to sI/R condition and we found SB203580 showed cardioprotective effect on cardiac hyperglycaemia subjected to sI/R condition.

In 2013, Jingfu Chen and colleagues have been reported that the high-glucose concentration could induce ROS production in H9c2 cell leading to cell injury and this situation was alleviated by SB203580 treatment (269). The effective of attenuation ROS induce H9c2 cell injury in high-glucose condition of SB203580 treatment also reported by the study of Yiyan Lei and colleagues (270). Moreover, it has been demonstrated that high-glucose condition could induce intracellular ROS in cardiomyocytes and treatment with SB203580 could reduce intracellular ROS production through ROS-MAPK-NF-κB signalling pathway (271). In addition, the effective of another p38 MAPK inhibitor (SB 202190) to reducing ROS production in high-glucose condition was investigated (272). From these evidences similar to our finding, we found that treatment of H9c2 cell with SB203580 in high-glucose condition could improve intracellular ROS generation.

As previously mentioned, the metformin and SB203580 could reduce cardiac cell death in hyperglycaemic condition. In this study we hypothesized that combination between metformin and SB203580 could improve cardiac cell death in hyperglycaemic condition. The results showed that combination between metformin and SB203580 did not affect to cell viability of H9c2 cell, which induced hyperglycaemic condition. From this result may be due to no toxicity from high glucose in H9c2 cell. However, treatment with combine drugs did not toxicity in hyperglycaemic condition. This finding in contrast to hyperglycaemia subjected to sI/R experiment, the result indicated that

treatment with combination between metformin and SB203580 improve cardiac cell death in hyperglycaemia subjected to sI/R condition. In the part of ROS production, treatment with combine drugs could non-significantly reduce high-glucose induce ROS production when compare to SB203580 treatment alone. From this situation may be due to dose of metformin combine to SB203580 had interfered each other. Therefore, the optimization dose of combine drugs was further investigated for taking the best advantage of combine drugs treatment.

**Determination of diabetic condition in animal model.**

In this study, the Goto-Kakizaki rat (GK rat), which is non-obese spontaneous type 2 diabetes, was performed as a diabetic model. The Wistar rats (Control non-diabetic group) and GK rats (Diabetic group) were measured the body weight, fasting blood glucose level, Haemoglobin $A_{1c}$ ($HbA_{1C}$) and oral glucose tolerance test (OGTT) for diagnosis of type 2 diabetic-like condition by using the human criteria that announced by American Diabetes Association(ADA) guideline in 2017 (241), due to lack of diagnostic criteria for diabetic in laboratory animal.

**1. Confirmation of lean type2 diabetic animal model.**

1.1 Determination of body weight of lean type 2 diabetic model.

The Wistar rats and GK rats were measured the body weight and the growth rate at age of 7, 15 and 20 weeks for verification of lean type 2 diabetic model. The result showed that the body weight of Wistar rats at 7 weeks old was 178.2 ± 12.80 g, at 15 weeks old was 421.7 ± 47.91 g and at 20 weeks old was 463.7 ± 46.35 g. The body weight of GK rats at 7, 15 and 20 weeks old were 140.6 ± 10.15 g, 297.6 ± 12.97 g and 315.3 ± 14.51 g, respectively. To determine the actual growth rate of the animal, the growth rate of body weight was assessed by plotting the linear graph and calculated for the linear regression equation analysis. The result showed that the linear equation of Wistar rat and GK rat was $y = 22.95x - 1.758$ and $y = 14.02x - 0.4524$, respectively (Figure 23). From these results indicated that the mean body weight of Wistar rat was higher than GK rat and the slope of linear equation of Wistar rat was greater than GK rat. Therefore, the GK rats have a lean phenotype.

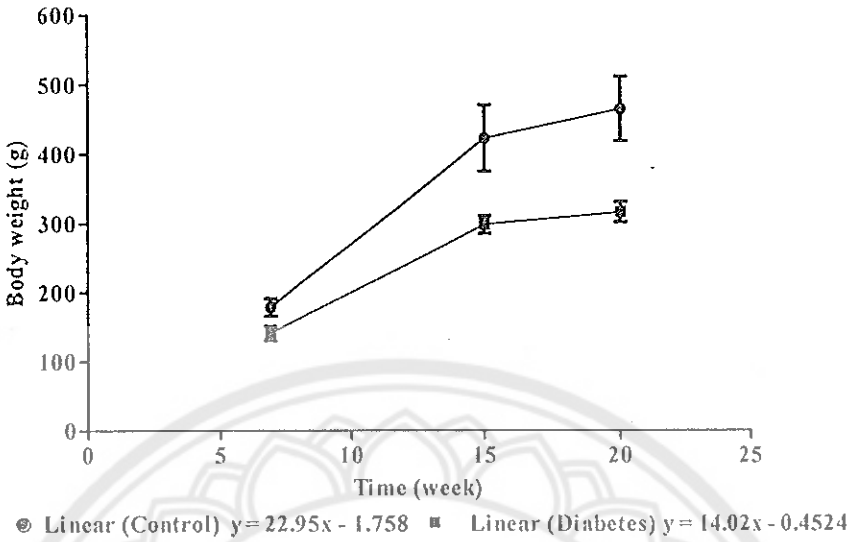


**Figure 23 Determination of lean type 2 diabetic model by using the growth rate at week 7, week 14 and week 20. All rats were measured body weight at week 7, week 14 and week 20. Then, the body weight was analysed the growth rate by linear regression analysis (n=10, n=40).**

Control (Wistar rats) Diabetes (GK rats)

**2. Diagnosis of type 2 diabetic-like condition in animal model.**

2.1 Determination of fasting blood glucose in animal model.

The fasting blood glucose level was measured after fasted rats for 12-14 h by using glucometer (SD GlucoNavii® GDH). The result showed that the fasting blood glucose level of Wistar rats were 84.00 ± 14.25 mg/dl. In GK rats, the fasting blood glucose level was 143.6 ± 32.00 mg/dl (Figure 24). This result suggested that the mean fasting blood glucose level of GK rat (Diabetic group) was significantly higher than Wistar rat (Control group). Moreover, the fasting blood glucose level of GK rat groups was greater than 126 mg/dl, which is one of the diagnostic criteria of diabetes in human announced by ADA.

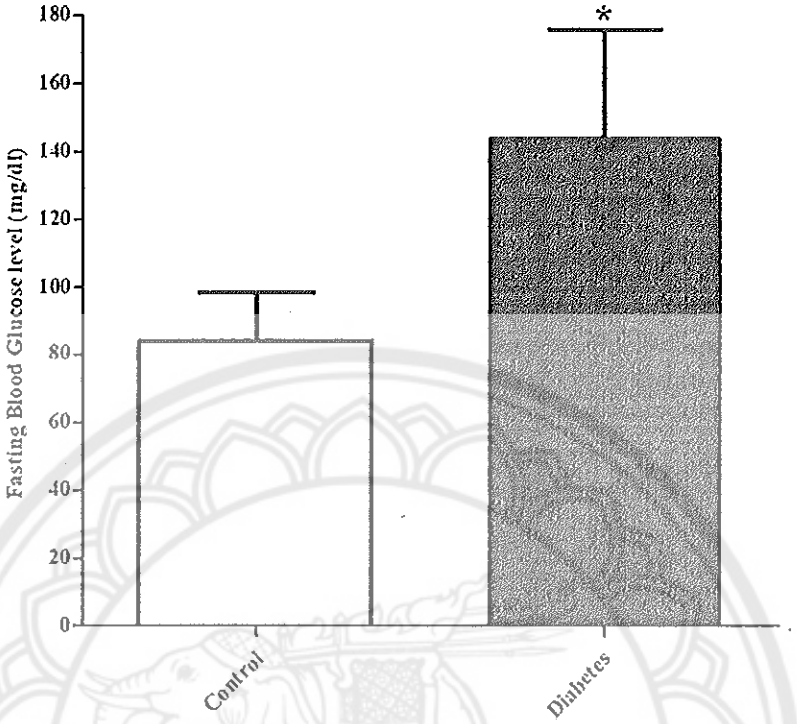


Figure 24 Determination the fasting blood glucose in animal model. Rats were measured blood glucose level, after fasted 12-14 h. Each bar graph represents means SD (n=10, n=40). *$p < 0.05$ vs control.

2.2 Determination of Haemoglobin $A_{1c}$ ($HbA_{1C}$) level in animal model.

For determination of percentage of $HbA_{1c}$, all rats were collected blood from tail vein and analysed by CLOVER A1c™ Self-analyser. The mean percentage of $HbA_{1c}$ level of GK rat was 6.532 ± 0.56 % that higher than the mean percentage $HbA_{1c}$ level of Wistar rat (4.120 ± 0.12 %) (Figure 25). The result indicated that the mean percentage $HbA_{1c}$ level of GK rat was higher than Wistar rat. Moreover, the percentage of $HbA_{1c}$ of GK rat groups was greater than 6.5 %, which is one of the diagnostic criteria of diabetes in human announced by ADA.

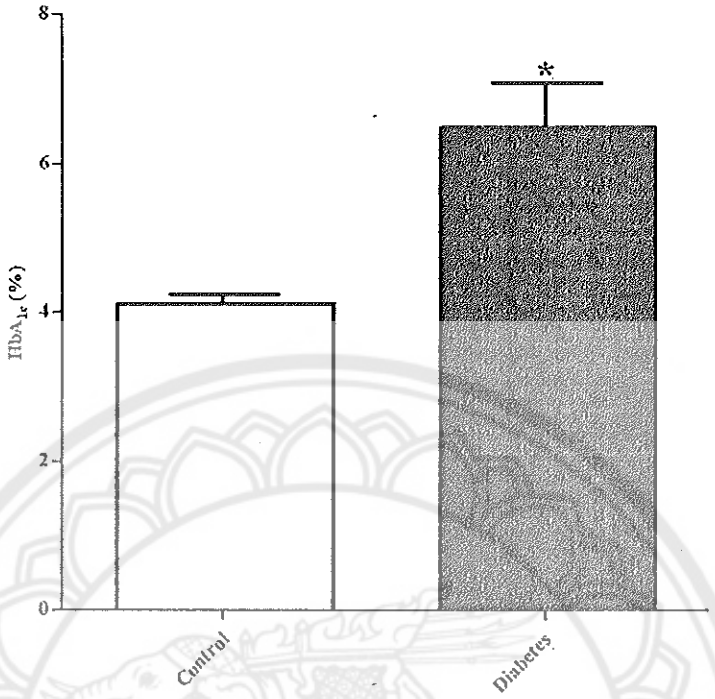


**Figure 25 Determination of Haemoglobin $A_{1c}$ level in animal model. The tail vein blood of rats was measured the percentage $HbA_{1c}$ level. Each bar graph represents means SD (n=10, n=40). *$p < 0.05$ vs control.**

2.3 Determination of oral glucose tolerance test (OGTT) in animal model.

The oral glucose tolerance test was performed by assessing blood glucose level after fasted rats for 12-14 h, and then treatment with 75 g glucose. The blood glucose was collected and measured after 30 min to 2 h. The result showed that in the Wistar rat blood glucose level at 2 h after treated with 75 g glucose was 111.7 ± 14.53 mg/dl and in GK rat was 265.6 ± 59.45 mg/dl (Figure 26). The result suggested that the blood glucose level in GK rat was higher than 200 mg/dl, which are the ADA criteria for diagnosis of human diabetes.

From these findings including body weight, fasting blood glucose and OGTT indicated that the GK rats in this study show a non-obese diabetic-like phenotype.

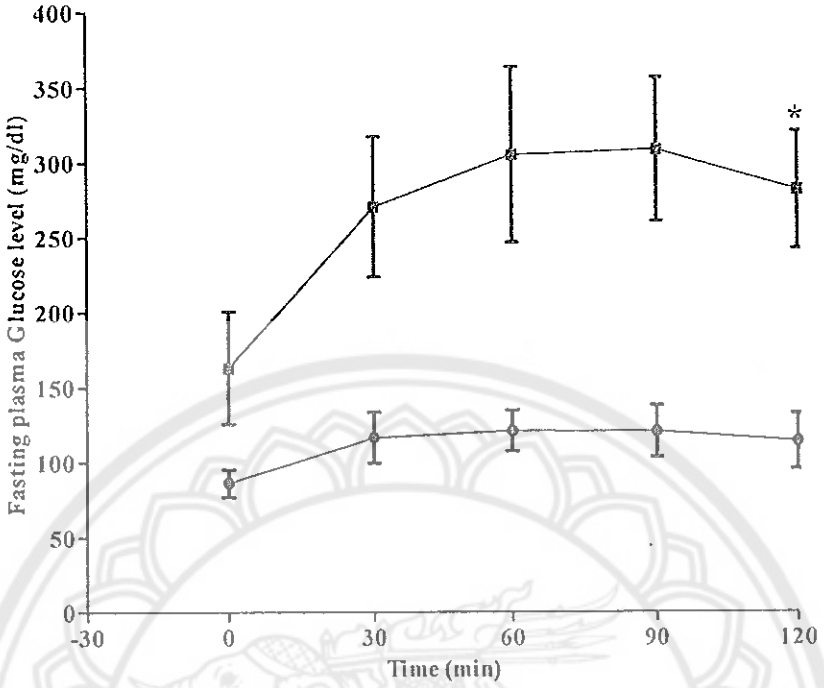


Figure 26 Determination of oral glucose tolerance test in animal model. After fasting, the rats were treated with 75 g of glucose. Then, the blood glucose was measured after 30 min to 2 h. Each line graph represents means SD (n=10, n=40). *$p < 0.05$ vs control.

After the GK rat was diagnosed with lean type 2 diabetic-like phenotype, all of rats were divided into 5 groups including control group (Wistar rat), diabetic group (GK rat), metformin group (GK rat), SB203580 group (GK rat) and combination group (GK rat). Then the effect of metformin, SB203580 and combination between metformin and SB203580 to improve diabetic parameters including fasting blood glucose and $HbA_{1c}$ were investigated.

**Determination of fasting blood glucose after treatment in animal model.**

**1. Determination effect of metformin on fasting blood glucose in diabetes rats.**

The rats were treated with 15 mg/kg of metformin for 4 weeks. Then, the blood was collected after fasted 12-14 h for determination of blood glucose level. The result showed that the fasting blood glucose level was 159.7 ± 48.45 mg/dl and 113.3 ± 9.46 mg/dl in before treatment and after treatment, respectively (Figure 27). The data analysis showed that after 4 weeks of metformin treatment the fasting blood glucose level was significantly decreased when compared to before treatment ($p<0.05$). This result indicated that treatment with metformin could reduce the fasting blood glucose in diabetic rat.

**2. Determination effect of SB203580 on fasting blood glucose in diabetes rats.**

After 4 weeks of treated with 2 mg/kg SB203580, the fasting blood glucose level of diabetic rat, before treatment, was significantly higher than the control group (148.6 ± 33.48 mg/dl vs 84.00 ± 14.25 mg/dl; $p<0.05$) and treatment with SB203580 was significantly decreased blood glucose level when compared to before treatment (106.9 ± 13.80 mg/dl vs 148.6 ± 33.48 mg/dl; $p<0.05$) (Figure 27). The result indicated that treatment with SB203580 could improve the fasting blood glucose level in diabetic rat.

**3. Determination effect of combination between metformin and SB203580 on fasting blood glucose in diabetes rats.**

The fasting blood glucose level, after treatment with combination between 15 mg/kg metformin and 2 mg/kg SB203580 for 4 weeks was determined. The result showed that fasting blood glucose level in diabetic rat, before treatment, was

significantly increased when compare to control group (152.5 ± 39.60 mg/dl vs 84.00 ± 14.25 mg/dl; $p<0.05$) and treatment with combination drugs was significantly decreased fasting blood glucose level when compared to before treatment (113.2 ± 11.97 mg/dl vs 152.5 ± 39.60 mg/dl; $p<0.05$) (Figure 27). This result suggested that the combination between metformin and SB203580 could improve the fasting blood glucose in diabetic rat. However, there were no further any affect between metformin and SB203580 to reduce the fasting blood glucose level in diabetic rat.

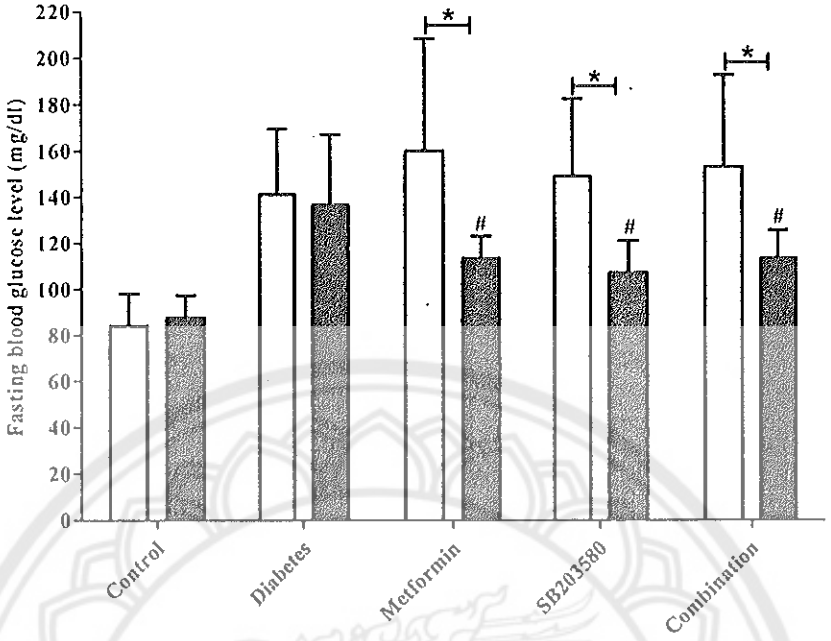


**Figure 27 Determination of fasting blood glucose after treatment in animal model. The rats were treated with combine drug (metformin and SB203580) for 4 weeks. Then, the blood glucose level was measured after 12-14 h fasting. Each bar graph represents means SD (n=10).** $^{*}p < 0.05$ **vs Before treatment,** $^{\#}p < 0.05$ **vs diabetes.**

**Determination of Haemoglobin $A_{1c}$ ($HbA_{1C}$) after treatment in animal model.**

**1. Determination effect of metformin on Haemoglobin $A_{1c}$ ($HbA_{1C}$) in diabetes rats.**

The results of $HbA_{1c}$ level were also assessed after treatment with each drug for 4 weeks. The percentage $HbA_{1c}$ of diabetic rat, before treatment, was significantly higher than the control group (6.58 ± 0.54 % vs 4.12 ± 0.12 %; $p<0.05$). Treatment with metformin was significantly decreased the percentage $HbA_{1c}$ from 6.50 ± 0.51 % to 5.36 ± 0.71 ($p<0.05$) when compared to before treatment (Figure 28). This result demonstrated that treatment with metformin in diabetic rat could improve $HbA_{1c}$ level.

**2. Determination effect of SB203580 on Haemoglobin $A_{1c}$ ($HbA_{1C}$) in diabetes rats.**

In SB203580 treatment, the result showed that the percentage $HbA_{1c}$ level in before treatment and after treatment of SB203580 were increased when compare to control group (6.93 ± 0.53 % and 6.13 ± 0.48 % vs 4.12 ± 0.12 %). However, treatment of SB203580 in diabetic rat was significantly decreased the percentage $HbA_{1c}$ from 6.93 ± 0.53 % to 6.13 ± 0.48 % ($p<0.05$) (Figure 28). The result indicated that SB203580 could reduce the $HbA_{1c}$ level in diabetic rat.

**3. Determination effect of combination between metformin and SB203580 on Haemoglobin $A_{1c}$ ($HbA_{1C}$) in diabetes rats.**

After treatment with combination drugs the $HbA_{1c}$ level of diabetic group and combination group, before treatment, were increased when compared to control group (6.58 ± 0.54 % and 6.66 ± 0.70 % vs 4.12 ± 0.12 %). Moreover, treatment with combination drug was significantly decreased the percentage $HbA_{1c}$ level from 6.66 ± 0.70 % to 5.03 ± 0.30 %; ($p<0.05$) when compared to before treatment (Figure 28). From this result suggested that treatment with combination between metformin and SB203580 in diabetic rat could reduce the $HbA_{1c}$ level and the combination of drugs did not provide a further significant reduction in the $HbA_{1c}$ level.

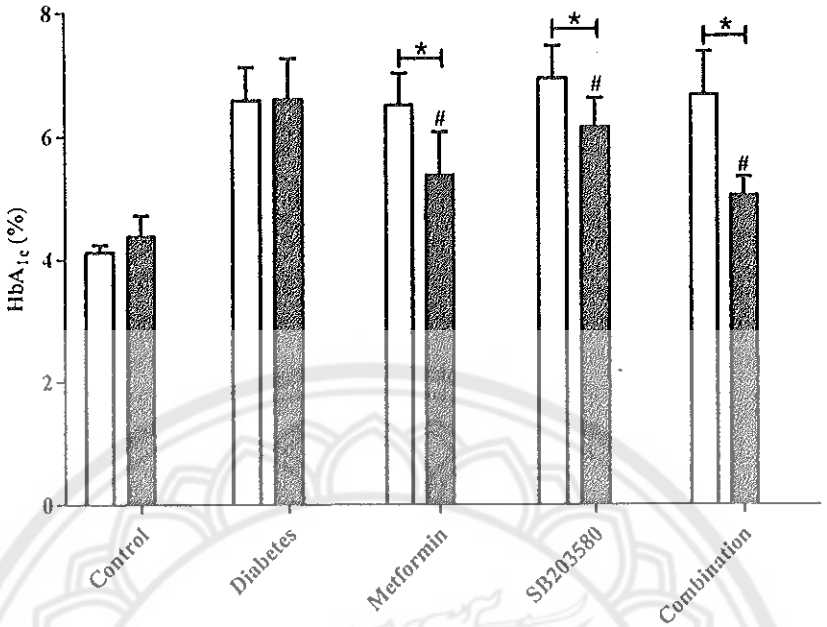


**Figure 28 Determination of Hb $A_{1c}$ level after treatment with metformin or SB203580 or the combination between metformin and SB203580. After treatment for 4 Weeks, the tail vein blood of rats was measured the percentage $HbA_{1c}$ level. Each bar graph represents means SD (n=10). *$p < 0.05$ vs Before treatment, #$p < 0.05$ vs diabetes.**

**Determination of plasma insulin level after treatment in animal model.**

The plasma insulin level was performed after 4 weeks of treatment by ELISA kit. The results showed that all of diabetic groups, including untreated and treated group, were significantly decreased plasma insulin level when compared to control group (18.07 ± 6.16 ng/ml vs 8.78 ± 2.72 ng/ml, 5.07 ± 3.84 ng/ml, 9.67 ± 3.97 ng/ml and 6.34 ± 3.82 ng/ml) (Figure 29).

Treatment with metformin or combination drug could decrease the plasma insulin level when compared to diabetic group (5.07 ± 3.84 ng/ml or .34 ± 3.82 ng/ml vs 8.78 ± 2.72 ng/ml). In contrast to SB203580 treatment, the plasma insulin level was increased when compare to metformin group and combination group and did not significantly difference in diabetic group. However, treatment with SB203580 did not provide a further reduction in plasma insulin level in combination with metformin (Figure 29).

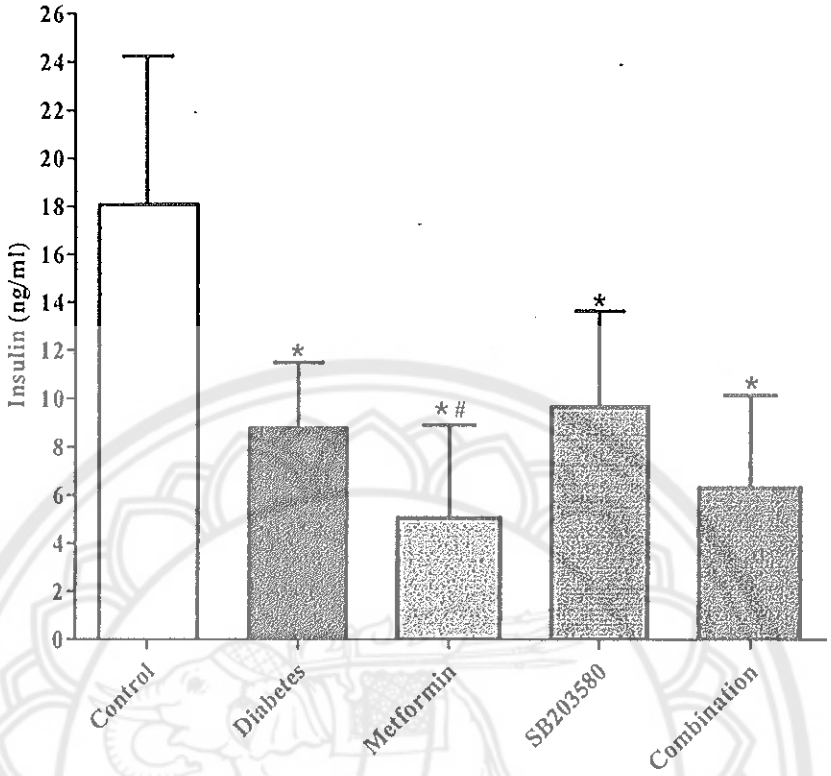


**Figure 29 Determination of plasma insulin level after treatment in animal model. The plasma was collected after 4 weeks of treatment and assessed plasma insulin level by ELISA kit. Each bar graph represents means SD (n=10). *$p < 0.05$ vs control, $^{\#}p < 0.05$ vs among groups.**

**Determination of cardiac function after treatment in animal model.**

In cardiac function, all rats were treated with metformin or SB203580 or metformin combined SB203580 for 4 weeks. Then, the rats were measured cardiac function parameter by using echocardiography as showed in figure 30. The results showed that, in the GK rats (Diabetic group), left ventricular internal dimension at end-diastole (LVIDd) and left ventricular internal dimension at end-systole (LVIDs) were significantly decreased (0.387 ± 0.04 cm vs 0.655 ± 0.09 cm and 0.248 ± 0.05 cm vs 0.432 ± 0.09 cm, respectively; $p<0.05$). However, the results showed that left ventricular posterior wall thickness at end-diastole (LVPWd) and left ventricular posterior wall thickness at end-systole (LVPWs) were stabilized when compared to Wistar rats (Control group) (0.265 ± 0.01 cm vs 0.285 ± 0.05 cm and 0.329 ± 0.10 cm vs 0.319 ± 0.05 cm, respectively), it means the heart wall was thickness.

Moreover, both end-diastolic volume (EDV) and end-systolic volume (ESV) were significantly reduced when compared to Wistar rat (0.152 ± 0.05 ml vs 0.694 ± 0.24 ml and 0.048 ± 0.02 ml vs 0.221 ± 0.09 ml, respectively; $p<0.05$). This resulted in a significant reduction of the blood flow and blood volume pumped per beat, which measured by stroke volume (SV) (0.105 ± 0.03 ml vs 0.473 ± 0.17 ml; $p<0.05$) and cardiac output (CO) (0.050 ± 0.01 L/min vs 0.169 ± 0.07 L/min; $p<0.05$). In addition, the heart rate (HR) and the ejection fraction (EF) percentage of GK rat were significantly increased when compared to Wistar rat (479.7 ± 60.46 Bpm vs 354.4 ± 25.97 Bpm and 70.68 ± 7.64 % vs 67.78 ± 7.67 %, respectively; $p<0.05$).

The combination of metformin and SB203580 could significantly increase EDV (0.387 ± 0.13 ml; $p<0.05$) and increase ESV (0.084 ± 0.06 ml) when compared to diabetic group. In addition, both SV and CO were significantly increased in combine drugs treated when compare to diabetic group (0.304 ± 0.08 ml vs 0.105 ± 0.03 ml and 0.130 ± 0.03 L/min vs 0.050 ± 0.01 L/min, respectively; $p<0.05$) whereas the heart rate was reduced when compared to diabetic group (432.4 ± 37.66 Bpm vs 479.7 ± 60.46 Bpm). From these results demonstrated that in the GK rats in this study displayed hypertrophic cardiomyopathy and treatment with combination between metformin and SB203580 could improve cardiac function in diabetic condition.

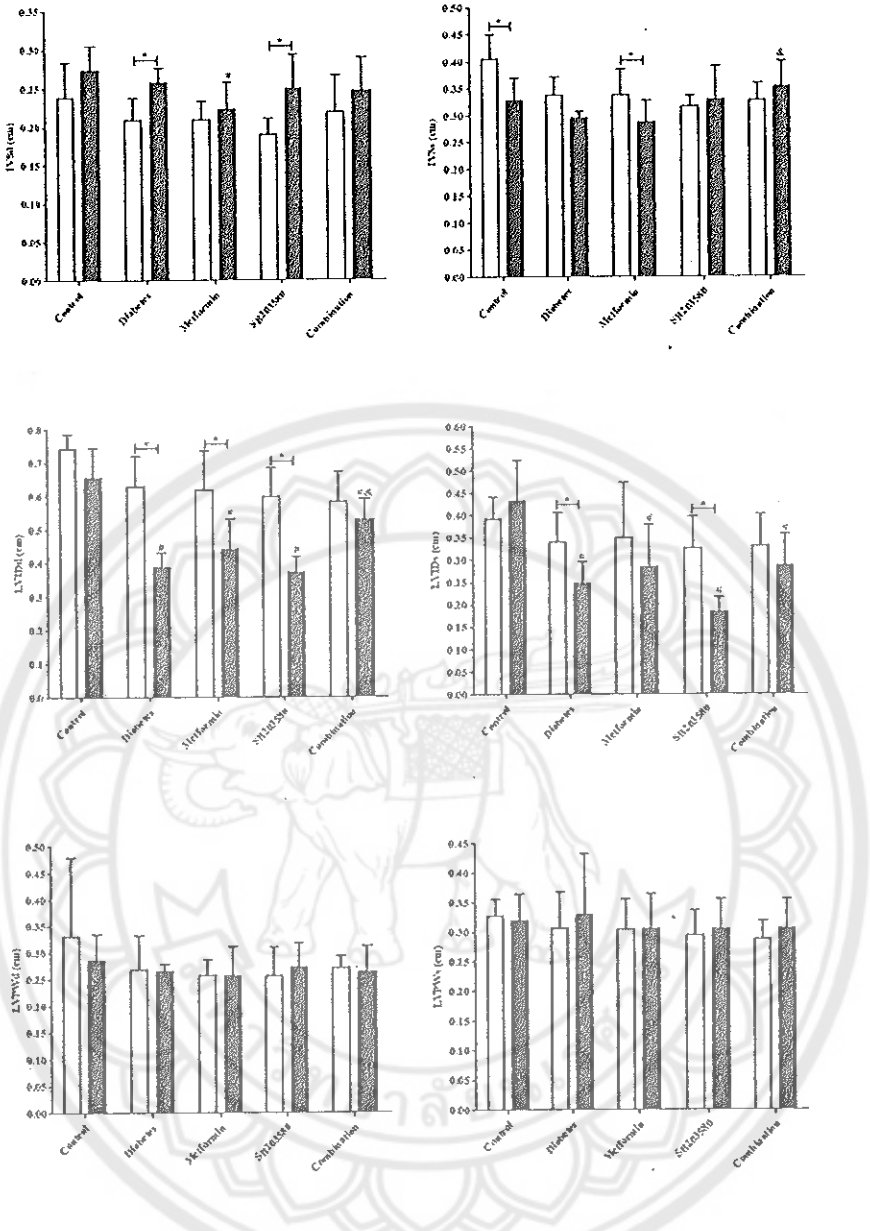

**Figure 30 Cardiac function parameters measured by echocardiography in animal model. After 4 weeks of treatment, all of rats were measured cardiac function parameters. Each bar graph represents means SD (n=10). *$p < 0.05$ vs among before and after treatment, $^{\#}p < 0.05$ vs control, $^{\&}p < 0.05$ vs diabetes.**

Before Treatment After Treatment

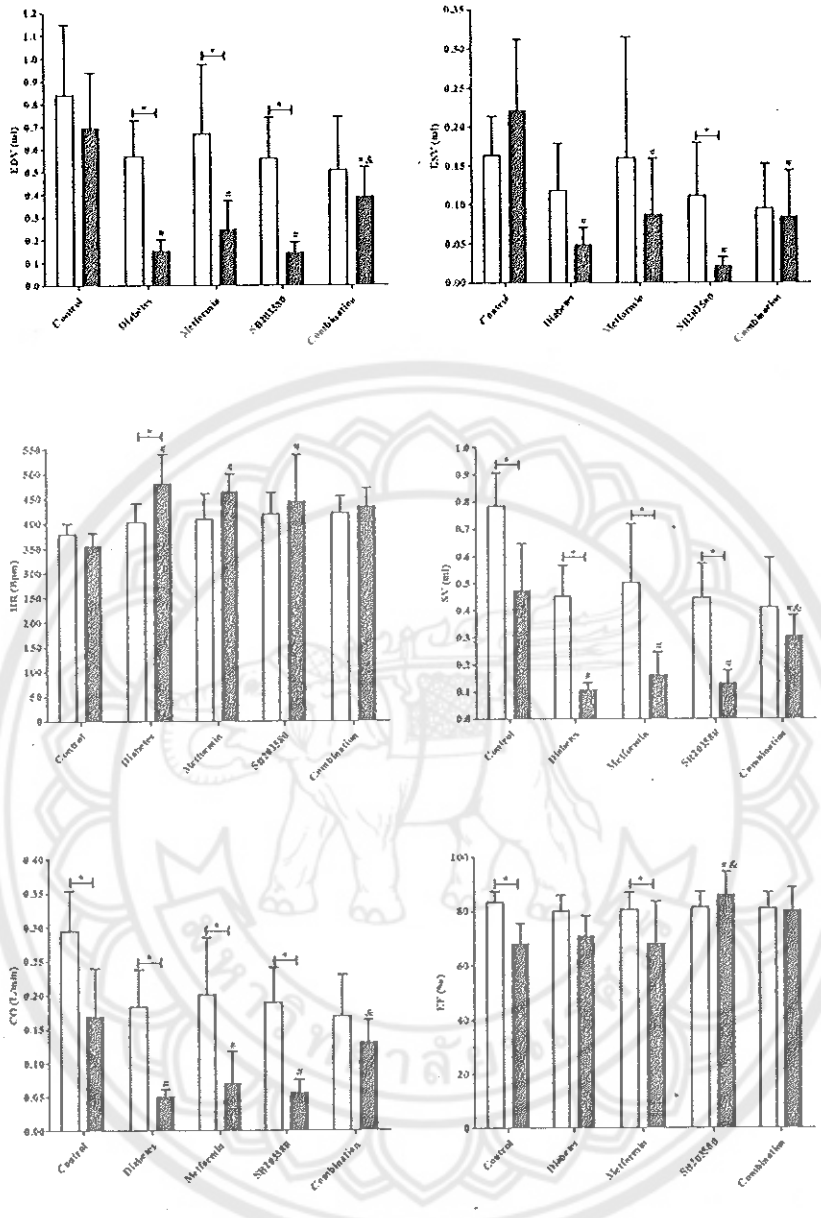

Figure 30 (cont.)

Type 2 diabetes is a non-communicable disease that causes major global health problems. Most of diabetic studies have been performed in animal models including obese models and non-obese models (273). The major cause of diabetes associated with obesity. However, it has been several reported that mostly Asian population, which is non-obesity, has high prevalence in type 2 diabetes (232, 274, 275). The Goto-Kakizaki rat (GK rat) is spontaneous type 2 diabetes non-obese model. The GK rats have been presented an impaired glucose tolerance at early of age leading to moderate insulin resistance, finally result in diabetic condition. Moreover, the GK rats could display diabetic complications at 12 weeks of age. These diabetic characteristics that exhibition in GK rats similar to the diabetic progression in human. Therefore, in an *in vivo* drug treatment in this study, a non-obese spontaneous type 2 diabetes Goto-Kakizaki rat (GK rat) was used as a diabetic model. Firstly, the phenotype of the rats was confirmed of lean type 2 diabetic-like model.

For confirmation of lean model, most of the previous studies were performed by using the body weight and they found that the GK rats, as lean model, with a body weight less than the matched control group, or normal model (276, 277). Similar to this study, the growth rate (Body weight) was determined for confirmation of lean model and the results found that the body weight of GK rat was less than the Wistar rat. From this finding could indicate that the GK rat has a lean phenotype. However, the body weight assessment alone may be insufficient and not accurate for lean model determination. In fact, previously evidences were determined lean model by using many parameters including body weight, food intake, and longitudinal bone, the length of the body and the length of the tail. Therefore, other parameters need to be further investigated (277-279).

In the confirmation type 2 diabetic-like model, the previously studies demonstrated that the criteria for diabetic diagnosis was performed by using the fasting blood glucose level or OGTT or both of fasting blood glucose level and OGTT. The previous findings showed that the fasting blood glucose level and OGTT in GK rat was higher than control rat (276, 280, 281). According to the lack of diagnostic criteria for diabetes in animal, our study was decide to use the human diabetic criteria based on the American Diabetes Association (ADA) guideline 2017 (241). These parameters include

fasting blood glucose level or $HbA_{1c}$ level or OGTT. Our results showed that the GK rat met all the criteria and hence demonstrated diabetic-like phenotype.

After that, the effect of anti-diabetic drug (metformin), p38 MAPK inhibitor (SB203580) and combination drug were determined. The results showed that treatment with all drugs could significantly decrease diabetic criteria such as fasting blood glucose level and $HbA_{1c}$ level, when compared to diabetic group. Although the combination between metformin and SB203580 did not further improve diabetic parameters but combining metformin and SB203580 did not interfere with the effectiveness of each other to improve diabetic parameters.

In 2009, Taishi Yoshida and colleagues was reported that metformin could reduce plasma glucose level in the GK rat by increasing glucose uptake (282). Moreover, treatment with metformin in GK rat could improve glycation, decrease hepatic gluconeogenesis and improve insulin sensitivity by increasing insulin stimulated peripheral glucose uptake but not change or increase the insulin secretory responses (283-285). In this study, the reducing blood glucose level and improve insulin sensitivity with did not increase plasma insulin secretory in GK rat was also found after metformin treatment. However, our study the effect of metformin on plasma insulin level was only determined. In the assessment of insulin sensitivity need to be investigated for confirmation the potential of metformin in improvement of insulin sensitivity.

The previous study indicated that p38 MAPK could increase activity by insulin resistance or type 2 diabetes, which cause of worsen effect in type 2 diabetes condition (286). Therefore, many studies attempt to inhibit p38 MAPK activation, by using p38 MAPK inhibitor. It has also reported that inhibition of p38 MAPK by selective inhibitor, could reduce glucose level via increasing the insulin-stimulated GLUT4 expression on membrane, which resulting in increased glucose uptake (287, 288). Moreover, Burns CJ. and colleagues has been reported that inhibition of p38 MAPK by SB203580 in rat islets of Langerhans, which stimulated glucose condition, did not significant effect on the stimulation of insulin secretion (289). These evidences have been reported that the effect of SB203580 treatment on high-glucose or hyperglycaemic condition in cellular responses (*in vitro*), however the effect of SB203580 treatment on diabetic condition in *in vivo* response have not been investigated. It has been known that SB203580 treatment on diabetes or hyperglycaemic condition were associated with cardioprotection (17,

225, 226). It seems like our study is the first to determine the effective glycaemic parameters (fasting blood glucose, $HbA_{1c}$ level, plasma insulin level) of SB203580 treatment in diabetic animal model. The findings from our study also suggested that treatment of p38 MAPK inhibitor (SB203580) could reduce of blood glucose level, reduce percentage $HbA_{1c}$ level and did not affect in plasma insulin secretion response.

Then, the effect of combination between metformin and SB203580 on improving diabetic parameters were determined. The results suggested that treatment with combine drugs in diabetic rat could improve diabetic parameters, including reduced blood glucose level, reduced $HbA_{1c}$ level and decreased plasma insulin level. However, these results did not provide a further reduction to improve diabetic parameters between metformin and SB203580 combination. These results indicated that metformin and SB203580 did not interfere with each other to improve diabetic parameters. However, in this study the effect of combination between metformin and SB203580 was only determined of diabetic parameters. In the drugs interaction and drug toxicity in hyperglycaemic condition need to be investigated for a precise therapeutic potential of metformin and SB203580 in diabetes or diabetic complication disease, particularly cardiovascular disease.

The progression of hyperglycaemic condition is a cause of complication especially, cardiovascular disease (CVD), which is one of diabetic cardiomyopathy, result of cardiac dysfunction. In previously evidence has been reported that 52% of type 2 diabetic patients was death by CVD and 44% in type 1 diabetes patients (290). Moreover, it has been reported that the characteristic of diabetic cardiomyopathy is left ventricular hypertrophy and contractile dysfunction (291). In addition, it has been reported that the diabetic animal models could demonstrate difference cardiovascular changes (292). In the GK rat also have been reported that the model could display pathology of cardiac dysfunction, including left ventricle remodelling with marked hypertrophy, increased extracellular matrix deposition and mild hypertension (293). In 2008, Erik Vahtola and colleagues have been demonstrated that the 12-week-old GK rat was displayed in cardiac hypertrophy and systolic dysfunction, which assessment by increasing of heart weight-to-body weight ratio, left ventricle diameter at diastole, left ventricular posterior wall thickness at diastole, reducing of cardiac output and heart rate (294). These were contrary to our findings. The results from our study found that the

20-week-old GK rat showed cardiac hypertrophy that is marked by stable left ventricular posterior wall thickness at end-diastole (LVPWd), reducing of cardiac output and increasing of heart rate. The differences of the results from our study and previous studies may be due to the age of rat, which related to the changing in cardiac function parameters. The age of the rat reported in our model was older than previous studies. This could lead to a higher degree of the development of the disease. However, in our study the heart weight-to-body weight ratio should be performed for confirming the hypertrophy.

**Determination effect of combination between metformin and SB203580 on infarct size by *ex vivo* Langendorff heart perfusion.**

After treatment for 4 weeks, rats were determined the effect of drugs by using percentage of infarct size, which was performed by Langendorff heart perfusion system. The result showed that the percentage infarct size of diabetic group was significantly increased when compared to control group (59.73 ± 5.20 % vs 43.41 ± 8.00 %, $p$ <0.05). Treatment with metformin or SB203580 or combination drug in diabetic rats was significantly decreased percentage infarct size from 59.73 ± 5.20 % to 38.59 ± 8.03 %, 33.63 ± 7.63 %, 26.15 ± 3.69 %, respectively (Figure 31). Moreover, treatment with combination drugs was significantly decreased percentage infarct size when compared to metformin or SB203580 treated group. These results indicated that treatment with anti-diabetic drug or p38 MAPK inhibitor or combination of drugs could reduce cardiac death in diabetic rats, which have complicated with myocardial ischemia/reperfusion disease. In addition, treatment with combination between anti-diabetic drug and p38 MAPK inhibitor did not cause of interfere with the effectiveness of each other to reduce infarct size but it was enhanced efficiency each other to infarct size.

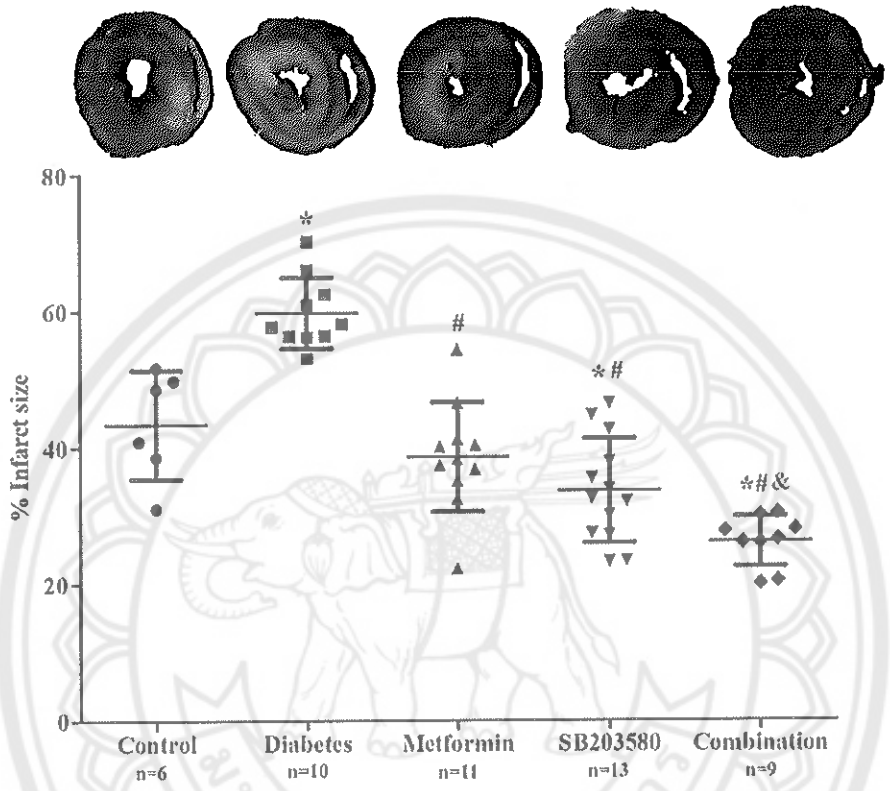


**Figure 1 Determination of infarct size after treatment in animal model. After 4 weeks of treatment, all rats were assessed infarct size by using Langendorff heart perfusion. Each bar graph represents means SD. *$p < 0.05$ vs control, $^{\#}p < 0.05$ *vs* diabetes, $^{\&}p < 0.05$ vs metformin or SB203580.**

The myocardial ischemia/reperfusion is an important cardiovascular diabetic complication disease, which a major cause of p38 MAPK activation resulting in apoptosis in heart of diabetic patient (21, 286). In previous study have been demonstrated that the diabetic heart could increase p38 MAPK activation by impairment of insulin signalling pathway and PI3K-Akt signalling, from this leading to aggravate lethal injury in diabetic heart. Moreover, hyperglycaemic condition could activate p38 MAPK activity through free fatty acid induce oxidative stress (ROS)- advanced glycation end products (AGEs) pathway (21). In addition, the increasing of p38 MAPK activation in diabetic heart could occur via inflammatory pathway (286). Therefore, the previous studies attempt to use anti-diabetic drug (metformin) or p38 MAPK inhibitor (SB203580) for preventing or improving glycaemic balance. For example, it have been reported that metformin demonstrated cardioprotective effect on myocardial ischemia/reperfusion injury in diabetic model and animal model by improving of cardiac function, reduced cell death, attenuated myocardial infarct size and improve ejection fraction (21, 91, 295). In p38 MAPK inhibitor (SB203580), the previous studies suggested that treatment with SB203580 in diabetic models could improve systolic function and improve cardiac contractile functions (6, 21). These findings were focused on the cardioprotective effect of metformin or SB203580 in diabetic model. However, the effect of metformin or SB203580 or combination drugs on ischemia/reperfusion injury of the diabetic heart has never been demonstrated. Therefore, in this study we determined the effect of drugs on ischemia/reperfusion injury of the diabetic heart by using infarct size analysis and we found that metformin or SB203580 or combination of metformin and SB203580 could reduce infarct size. Moreover, both of metformin and SB203580, which used in combination, did not interfere the effect of reducing infarct size with each other rather than they showed more effective for reducing infarct size. From this result could indicated that metformin or SB203580 show cardioprotective effect on diabetic heart with ischemia/reperfusion injury and they show the synergistic effects on diabetic heart with ischemia/reperfusion injury when used in combination.

Moreover, we were investigated the effect of metformin or SB203580 or combination between metformin and SB203580 in non-diabetic condition. In this experiment was performed by using the male C57BL/6 mice, which divide into 4 groups including, control group, metformin group, SB203580 group and combination group.

All of mice were treated with same concentration in the rat experiments for 4 weeks. In addition, the body weight and blood glucose level were measured every week throughout the experiment. Moreover, the mice were determined cardiac function and infarct size by Echocardiography and Langendorff heart perfusion system, respectively. The results showed that, the body weight, the blood glucose level and cardiac function did not statistic difference after treatment for 4 weeks. In the infarct size analysis, the result showed that the percentage of infarct size in SB203580 and combination treatment were significantly increased when compared to non-treatment (Data not showed). These results suggested that SB203580 or combination between metformin and SB203580 did not have cardioprotective effect on ischemia/reperfusion injury condition. Our findings was in contrary to previous studies, which indicated that SB203580 have cardioprotective effect on ischemia/reperfusion injury by reducing infarct size and improving cardiac function in myocardial infarction (MI) model (6). Although SB203580 have been reported for beneficial effect on ischemia/reperfusion injury, but also have some previous studies showed the discrepancy of the finding that SB203580 cause of failure to reduce the infarct size and abolish the cardioprotective effect of ischemic preconditioning (IPC) (6, 296). There are several reasons for explanation these inconsistencies including, species of model, dose, duration and protocol of treatment. However, our study was limited only infarct size investigation. Therefore, the mechanism of cellular transduction and the dose interaction need to be determined for potential therapeutic in cardiovascular disease.

**Determination of signal transduction in whole heart tissue responding to combinatorial agents by Western blotting.**

This experiment we hypothesized that the combination drugs reduced the apoptotic signalling activation (p38 MAPK, Bax, Caspase 3) and increased the survival signalling activation (Akt and BCl-2). The homogenate heart tissue from 5 groups were performed by Western blot analysis for determine protein phosphorylation.

In p38 MAPK phosphorylation, the result showed that diabetic group was significantly increased p38 MAPK phosphorylation when compare to control group ($1.20 \pm 0.09$ vs $0.82 \pm 0.04$, $p < 0.05$). Treatment with metformin or SB203580 or combination drugs were significantly decreased p38 MAPK phosphorylation when

compared to diabetes group (1.20 ± 0.09 vs 0.25 ± 0.09 or 0.34 ± 0.11 or 0.14 ± 0.14, $p < 0.05$). This result suggested that hyperglycaemic condition could induce the phosphorylation of p38 MAPK increasing and this p38 MAPK phosphorylation could reduce by metformin or SB203580 or combination between metformin and SB203580 treatment. However, treatment with SB203580 combined with metformin did not provide a further reduction in p38 MAPK phosphorylation (Figure 32).

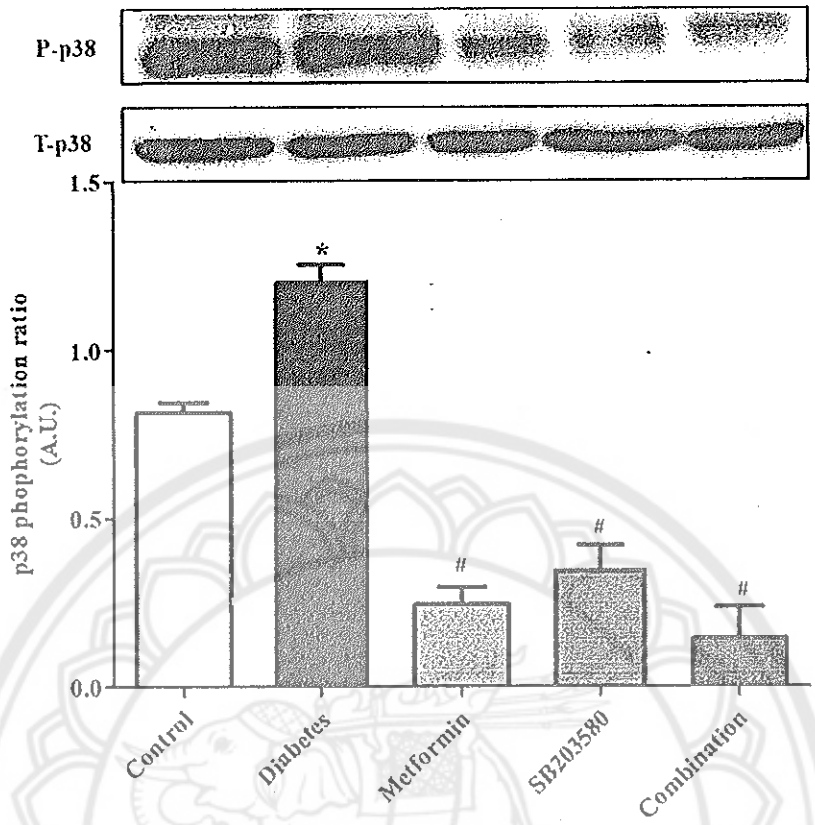


Figure 32 Determination of p38 MAPK phosphorylation in whole heart tissue by Western blot. After 4 weeks of treatment, the whole heart tissue homogenate was assessed p38 MAPK phosphorylation by using Western blotting analysis. Each bar graph represents means SD (n=3). $^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs diabetes.

In Akt phosphorylation, the result showed that the Akt phosphorylation did not different between control group and diabetic group (0.803 ± 0.32 and 0.792 ± 0.15). Treatment with drugs (Metformin or SB203580 or combined drugs) could increase the Akt phosphorylation when compared to diabetic group (0.792 ± 0.15 vs 1.23 ± 0.10, 1.06 ± 0.21, 0.914 ± 0.05). Especially, metformin treatment the result showed that Akt phosphorylation was significantly increased when compared to diabetic group. These results indicated that treatment with metformin or SB203580 in diabetic condition could increase the phosphorylation of Akt signalling rather than the combination between metformin and SB203580 treatment (Figure 33).

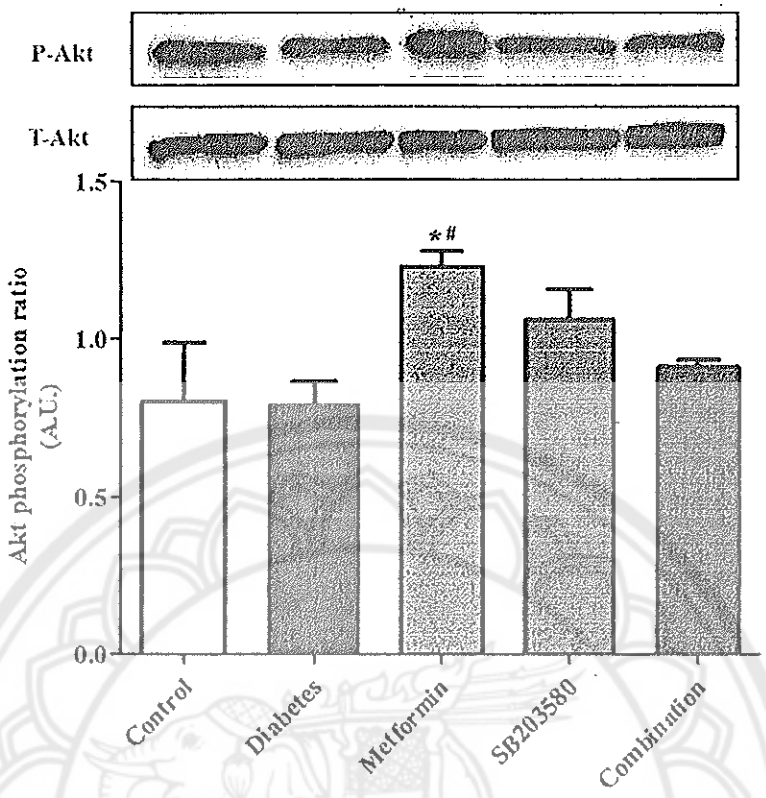


Figure 33 Determination of Akt phosphorylation in whole heart tissue by Western blot. After 4 weeks of treatment, the whole heart tissue homogenate was assessed Akt phosphorylation by using Western blotting analysis. Each bar graph represents means SD (n=3). $*p < 0.05$ vs control, $^{\#}p < 0.05$ vs diabetes.

Moreover, in this study we also determined the apoptosis pathway including Bcl-2, Bax and caspase 3. The result demonstrated that the diabetic group was significantly increased Bax/Bcl-2 ratio (0.59 ± 0.44 vs 0.32 ± 0.01, $p<0.05$; Figure 34) and significantly increased caspase 3 level (0.67 ± 0.23 vs 0.21 ± 0.04, $p<0.05$; Figure 35) when compared to control group. Treatment with metformin or SB203580 was significantly decreased Bax/Bcl-2 ratio (0.59 ± 0.44 vs 0.098 ± 0.04 or 0.099 ± 0.02, $p<0.05$; Figure 34) but not difference in caspase 3 level (0.51 ± 0.09 or 0.67 ± 0.09 vs 0.67 ± 0.23; Figure 35) when compared to diabetic group. In addition, treatment with combination between metformin and SB203580 was significantly reduced Bax/Bcl-2 ratio (0.59 ± 0.44 vs 0.14 ± 0.056, $p<0.05$; Figure 34) and caspase 3 level (0.67 ± 0.23 vs 0.17 ± 0.04, $p<0.05$; Figure 35) when compared to diabetic group.

These results indicated that diabetic completed with ischemia/reperfusion injury condition could induce cardiac cell death by increasing of p38 MAPK phosphorylation, BAx/Bcl-2 ratio, caspase 3 level and extenuating Akt phosphorylation. Treatment with combine drugs could improve cardiac cell death on diabetic completed with ischemia/reperfusion injury condition.

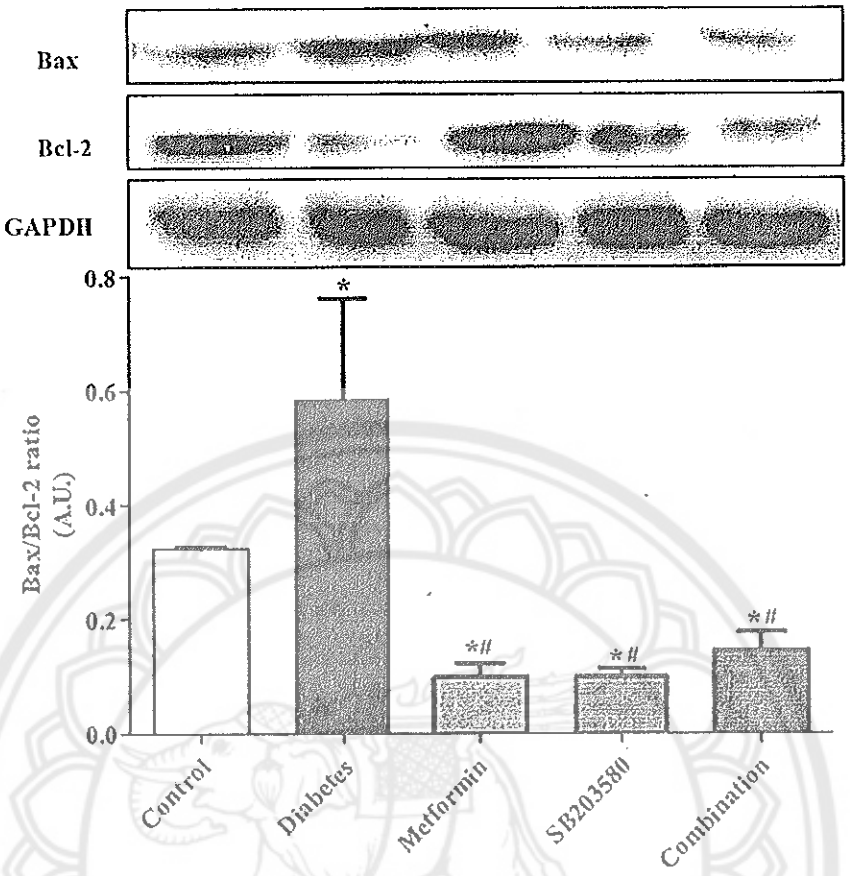


Figure 34 Determination of Bax/Bcl-2 ratio in whole heart tissue by Western blot. After 4 weeks of treatment, the whole heart tissue homogenate was assessed Bax and Bcl-2 level by using Western blotting analysis. Each bar graph represents means SD (n=3). $^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs diabetes.

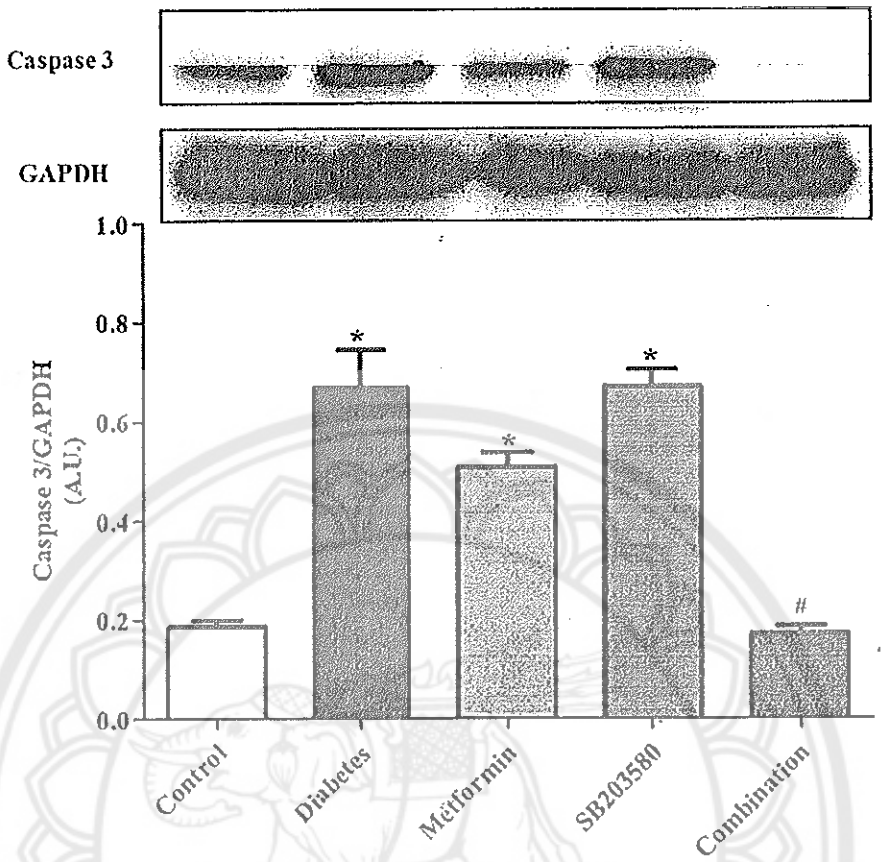


Figure 35 Determination of caspase 3 level in whole heart tissue by Western blot. After 4 weeks of treatment, the whole heart tissue homogenate was determined caspase 3 level by using Western blotting analysis. Each bar graph represents means SD (n=3). $^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs diabetes.

As previously reported that the diabetic condition or ischemia/reperfusion injury (I/R) condition induced the increasing of p38 MAPK phosphorylation, which cause of cardiac cell death by increasing of Bax/Bcl-2 ratio and caspase 3 level (297-299). In addition, the previous studies have been reported that treatment with p38 MAPK inhibitor (SB203580) (266, 268) or metformin (300, 301) could prevent cardiac cell death by inhibited p38 MAPK activation, attenuation of Bax/Bcl-2 ratio and reducing of caspase 3 level . This information shows only the effect of metformin or SB203580 on diabetes induced cardiac cell, however, the effect of combination between metformin and SB203580 on diabetes induced cardiac cell have not been investigated. So, we were hypothesized the diabetic combination with I/R condition leading to cardiac cell death worsening and this situation was recover by drugs (Metformin, SB203580, combine drugs) treatment. In this study the cardiac p38 MAPK phosphorylation, Akt phosphorylation, Bax/Bcl-2 ratio and caspase 3 level were determined by using Western blotting. Similar to previously reported, we found that stimulation of ischemia/reperfusion (I/R) injury in diabetic rats was induced cardiac cell death by increasing of p38 MAPK phosphorylation, Bax/Bcl-2 ratio, caspase 3 level more than control rats but not different of Akt phosphorylation. Treatment with the combination between metformin and SB203580 could improve diabetes induced cardiac cell death by inhibition of p38 MAPK phosphorylation, reducing of Bax/Bcl-2 ratio and decreasing of caspase 3 level. Interestingly, improvement diabetes induced cardiac cell death of the combination between metformin and SB203580 treatment did not associated with Akt phosphorylation. From this situation may be due to the effect of combination between metformin and SB203580 on diabetes induced cardiac cell death did not via Akt pathway or the dose of metformin combine to SB203580 had interfered each other. Therefore, the investigation of diabetes induced cardiac cell death other pathway or the optimization dose of combine drugs was further investigated for the best advantage of combined drugs treatment.

# CHAPTER V

# CONCLUSIONS

Type 2 diabetes mellitus is one of non-communicable diseases, which causes a major global health problem. It is characterized by impairment of insulin signalling leading to high blood glucose (hyperglycaemia). The detrimental effects of type 2 diabetes mellitus are not limited to metabolism, the progression of the disease also causes the complication to other organs, including heart. Diabetes mellitus is found to be associated with a 4-fold increased risk of acute myocardial infarction (AMI), which is a major cause of morbidity and mortality worldwide. Both AMI and diabetic condition are known to activate the basal p38 MAPK level, which aggravate cardiac cell injury and death in diabetic patient with AMI. Therefore, reduction of hyperglycaemic condition and p38 MAPK inhibition may possibly benefit to reduce myocardial damage in diabetic individual with AMI.

In this study, we assessed the combinatorial effect of SB203580, a p38 MAPK inhibitor, and metformin, a first line anti-diabetic drug, on myocardium under ischemia/reperfusion (I/R) with diabetic conditions.

We first investigate cardioprotective effect of SB203580 and metformin in *in vitro* using H9c2 cells, rat cardiac myoblast cells. H9c2 cells were induced to get hyperglycaemic condition by incubating with 33 mM D-glucose solution for 24 h and then incubate with SB203580 and/or metformin for 24 h. We found that hyperglycaemic condition did not cause cell death, but increased intracellular ROS generation. Treatment with either metformin or SB203580 or both drugs could reduce ROS production due to hyperglycemia. For the next experiment, hyperglycaemic H9c2 cells were subjected to simulated I/R. We found that hyperglycaemic condition aggravated I/R. Treatment with SB203580 or metformin or both drugs could reduce cardiac cell death due to hyperglycaemia with sI/R.

We then further investigate cardioprotective property of SB203580 and metformin in *in vivo* using Goto-Kakizaki (GK) rats as a non-obese spontaneous type 2 diabetes, and Wistar rats as a normoglycaemic control. Rats were divided into 5 groups

including control group, diabetic group, diabetes treated with metformin group, diabetes treated with SB203580 group, and diabetes treated with combination of SB203580 and metformin group. Diabetic parameters including fasting blood glucose (FBG) level, $HbA_{1c}$ and cardiac function were determined. After 4 weeks of treatment, the results showed that FBG and $HbA_{1c}$ levels in GK rat were significantly higher than the Wistar rat. Treatment with metformin or SB203580 or both drugs was significantly decreased the FBG and $HbA_{1c}$ levels when compared to the non-treated rats. For echocardiographic parameters, we found that heart wall thickness was significantly increase in GK rats compared with the Wistar rats. Treatment with the combination between metformin and SB203580 was significantly increased EDV, ESV, SV and CO when compared to non-treated rats. Taken together the GK rats displayed diabetic condition and hypertrophic cardiomyopathy. Treatment with the combination between metformin and SB203580 could improve diabetic parameters and cardiac function.

We then determine infarct limiting effect and cardioprotective signalling pathways. Langendorff heart perfusion system was used to induced global ischaemic and reperfusion condition. Percentage of infarct size and the cellular signalling were determined using TTC staining and Western blotting techniques, respectively. We found that diabetic condition could aggravate I/R injury by increasing of infarct size, p38 MAPK phosphorylation, Bax/Bcl-2 ratio and caspase 3 level but did not affect in Akt phosphorylation. Treatment with metformin or SB203580 could reduce infarct size, inhibited p38 MAPK phosphorylation, reduced Bax/Bcl-2 ratio, and increased Akt phosphorylation in diabetic rats. Treatment with combination between metformin and SB203580 could further reduce infarct size, inhibit p38 MAPK phosphorylation, decreased Bax/Bcl-2 ratio, and reduced caspase 3 expression, but did not affect phosphorylation of Akt. These results indicated that the diabetes concomitant with myocardial IR injury activate apoptotic signalling pathways and increase p38 MAPK phosphorylation, which lead to cardiac dysfunction and cardiac cell death. Combinatorial treatment between metformin and SB203580 provided cardioprotective properties as indicated by improved cardiac function and reduced myocardial death. The cardioprotective mechanisms were due to inhibiting p38 MAPK phosphorylation, and reduced apoptotic signalling pathways.

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# REFERENCES


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# APPENDIXES

# APPENDIXES

## SOLUTIONS

General laboratory chemicals were of analytical grade, solid chemicals were dissolved in DI $H_2O$ (resistivity > 18 mΩ/ $cm^3$) unless otherwise stated, and adjusted to the required pH with either HCl or NaOH.

### Tissue Culture

**1. Complete medium**

| | | |
|---|---|---|
| Dulbecco's Modified Eagles Medium (DMEM) | 500 | ml |
| Foetal Calf Serum | 50 | ml |
| Penicillin/Streptomycin (100 units/ml) | 5 | ml |

**2. Serum free medium**

| | | |
|---|---|---|
| Dulbecco's Modified Eagles Medium (DMEM) | 500 | ml |
| Penicillin/Streptomycin (100 units/ml) | 5 | ml |

**3. Phosphate-Buffered Saline (PBS)**

This was made from pre-formed tablets (GibcoBRL, UK) consisting of:

| | | |
|---|---|---|
| NaCl | 4.0 | g |
| KCl | 0.1 | g |
| $Na_2PO_4$ | 0.72 | g |
| $KH_2PO_4$ | 0.12 | g |

Dissolved in 500 ml DI $H_2O$.

**4. Trypsin solution**

| | | |
|---|---|---|
| Trypsin | 10 | ml |
| PBS | 40 | ml |

**Simulates Ischaemia**

**1. Basic Buffer (Stock)**

**Table 9 Solutions used to make Basic Buffer**

| Reagent | Required concentration (mM) | 1X (1 L) | 10X (200ml) |
|---|---|---|---|
| NaCl | 137 | 8.0 g | 16 g |
| KCl | 3.58 | 0.27 g | 0.54 g |
| $MgCl_2.6H_2O$ | 0.49 | 0.10 g | 0.2 g |
| $CaCl_2.2H_2O$ | 1.8 | 0.264 g | 0.528 g |
| HEPES | 4 | 0.953 g | 1.906 g |
| | | Adjust pH to 7.4 | |

**2. Control Buffer**

| | | |
|---|---|---|
| 10X Stock Basic Buffer | 5 | ml |
| Sterile DI $H_2O$ | 45 | ml |
| D-Glucose (20mM) | 0.225 | g |
| Na Pyruvate (1mM) | 5.5 | mg |

**3. Simulated Ischaemia (sI) Buffer**

| | | |
|---|---|---|
| 10X Stock Basic Buffer | 5 | ml |
| Sterile DI $H_2O$ | 45 | ml |
| 2-Deoxygluose (20 mM 2-DOG) | 82.1 | mg |
| Na Dithionite (1.0 mM) | 8.7 | mg |
| Na Lactate (30% = 1:2 dilution of 60% stock) | 172 | µl |

**Protein Harvesting Solutions**

**1. Sample Buffer (2X)**

| | | |
|---|---|---|
| Glycerol | 2.0 | ml |
| SDS | 6.0 | g |
| Tris | 1.4 | g |

To 100 ml with DI $H_2O$

Prior to use, add 100 µl of 2-mercaptoethanol (10%), 900µl of 2 X Sample Buffer and 5 µl of bromophenol blue (8% in ethanol)

**2. Lysis Buffer for tissue sample (50 ml)**

| | | |
|---|---|---|
| HEPES, pH 7.5 | 238.3 | mg |
| NaCl | 438.8 | mg |
| EDTA | 14.6 | mg |
| EGTA | 19.0 | mg |
| $Na_2VO_4$ | 9.20 | mg |
| NaF | 105.0 | mg |

Complete mini proteases inhibitor (1 tablet per 50ml of buffer)

To 50 ml with DI $H_2O$

**SDS-PAGE gel Solutions**

**1. Polyacrylamide gel solution**

1.1 Resolving gel (1.5 M Tris; 0.4% SDS; pH to 8.8 with HCl)

| | | |
|---|---|---|
| SDS | 2.0 | g |
| Tris | 90.9 | g |
| DI $H_2O$ | 300 | ml |

Adjust pH to 8.8 and add water to 500 ml

1.2 Stacking gel (0.5 M Tris; 0.4% SDS; pH to 6.8 with HCl)

| | | |
|---|---|---|
| SDS | 2.0 | g |
| Tris | 30.25 | g |
| DI $H_2O$ | 300 | ml |

Adjust pH to 6.8 and add add water to 500 ml

Polyacrylamide gels were made, depending on the concentration, according to table

**Table 10 Solutions used to make polyacrylamide gels for SDS-PAGE**

| Reagent | 7.5% gel | 10% gel | 12.5% gel | 15% gel | Stacking (4%) |
|---|---|---|---|---|---|
| MW range | | | 10-100 kDa | 5-70 kDa | |
| DI $H_2O$ | 9.0 ml | 5 (7.5) ml | 4 (6) ml | 3 (4.5) ml | 7 ml |
| 30% Acrylamide | 4.5 ml | 4 (6) ml | 5 (7.5) ml | 6 (9) ml | 2 ml |
| 1.5 M tris-HCl (pH8.8) | 4.5 ml | 3 (4.5) ml | 3 (4.5) ml | 3 (4.5) ml | - |
| 0.5 M tris-HCl (pH6.8) | - | - | - | - | 3 ml |
| 10% Ammonium persulfate | 90 µl | 60 (90) µl | 50 (75) µl | 50 (75) µl | 100µl |
| TEMED | 22.5 µl | 15 (22.5) µl | 15 (22.5) µl | 15 (22.5) µl | 10µl |

**2. Coomassie blue staining solution**

Protein can be visualised in polyacrylamide gels by exposure to coomassie blue solution followed by destaining to remove background staining.

| | | |
|---|---|---|
| Coomassie brilliant blue R250 | 0.25 | g |
| Methanol | 45 | ml |
| DI $H_2O$ | 45 | ml |
| Glacial acetic acid | 10 | ml |

**3. Destain solution**

| | | |
|---|---|---|
| Methanol | 400 | ml |
| $H_2O$ | 70 | ml |
| Glacial acetic acid | 530 | ml |

**4. Gel running buffer**

| | | |
|---|---|---|
| Tris | 30.3 | g |
| Glycine | 144.2 | g |
| SDS | 10.0 | g |

**5. Gel transfer buffer**

| | | |
|---|---|---|
| Tris | 30.3 | g |
| Glycine | 144.2 | g |

Add 100 ml of 10X Blotting Buffer to 200 ml of methanol and 700 ml of DI $H_2O$ to make 1L of 1x Blotting Buffer.

**Western blot Solutions**

**1. TBST buffer (10X)**

| | | |
|---|---|---|
| Tris | 24.2 | g |
| NaCl | 80 | g |

To make 1 x TBST, add 100 ml of 10X TBST to 900 ml of DI $H_2O$ and 1 ml of Tween-20

**2. Blocking buffer**

| | | |
|---|---|---|
| 10X TBST | 100 | ml |
| DI $H_2O$ | 900 | ml |
| Tween-20 | 1 | ml |
| Skimmed Milk powder | 5 | g |

**3. Antibody dilution buffer**

| | | |
|---|---|---|
| 10X TBST | 100 | ml |
| DI $H_2O$ | 900 | ml |
| Tween-20 | 1 | ml |
| Skimmed Milk powder | 1 | g |

**Langendorff-Perfusion**

**1. Anaesthetic solution**

All male rats were anesthetized by intraperitoneal injection with pentobarbital (300 mg/kg) and heparin (150 units)

**2. Modified Kreb-Henseleit Buffer**

**Table 11 Solutions used to make Modified Kreb-Henseleit Buffer**

| | Required Concentration(mM) | 5L | 2L | 1L |
|---|---|---|---|---|
| NaCl | 118.5 | 34.63 | 13.85 | 6.93 |
| $NaHCO_3$ | 25 | 10.5 | 4.20 | 2.10 |
| KCl | 4.7 | 1.77 | 0.71 | 0.35 |
| $MgSO_4.7H_2O$ | 1.2 | 1.47 | 0.59 | 0.29 |
| $KH_2PO_4$ | 1.2 | 0.8 | 0.32 | 0.16 |
| Glucose | 11 | 9.9 | 3.96 | 1.98 |
| Mix well , bubbled with 95% $O_2$+ 5% $CO_2$ ~ 5 mins | | | | |
| $CaCl_2.2H_2O$ | 1.4 | 1.04 | 0.42 | 0.21 |

**Note:** The buffer was filtered through a 0.8 μM cellulose nitrate micro-filter (Whatman, Maidstone, UK), and stored at 4°C for no longer than 1 day.

**3. Triphenyl tetrazolium chloride staining solution**

A 1% (w/v) triphenyl tetrazolium chloride (TTC) was used to demarcate the infarct zone in isolated mouse hearts.

| | | |
|---|---|---|
| TTC | 1 | g |
| PBS | 100 | ml |

**4. Rat heart fixative**

Following sectioning, the heart slices were fixed for 24 hours, prior to visualisation in a 10% (v/v) formaldehyde solution.

| | | |
|---|---|---|
| Formaldehyde (40%, v/v) | 12.5 | ml |
| $H_2O$ | 37.5 | ml |