THE STIMULATORY EFFECT OF PORCINE PLACENTA EXTRACT ON *IN VITRO* HIGH GLUCOSE-INDUCED ANGIOGENESIS DISTURBANCE

CHATCHAI NENSAT

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Biomedical Sciences - (Type A 2))
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Thesis entitled "The stimulatory effect of porcine placenta extract on *in vitro* high glucose-induced angiogenesis disturbance"

By CHATCHAI NENSAT

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Biomedical Sciences - (Type A 2) of Naresuan University

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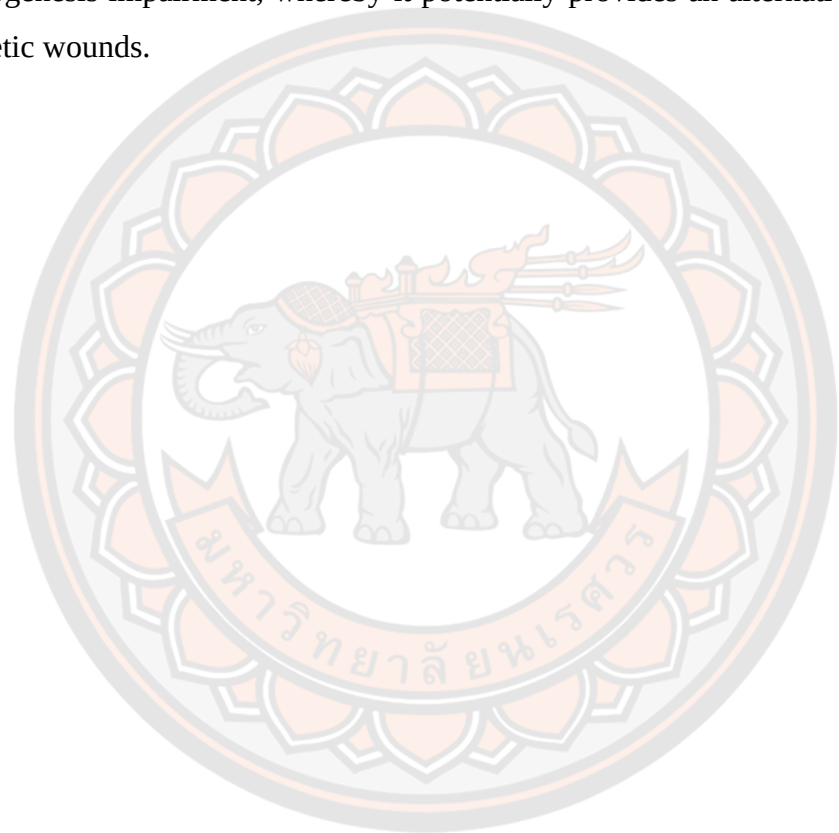
Keywords Porcine placenta extract, Angiogenesis impairment, Endothelial cell, Diabetes, Wound healing

ABSTRACT

High glucose (HG)-induced reactive oxygen species (ROS) overproduction impairs angiogenesis that is one pivotal factor of wound healing process. Angiogenesis impairment induces delayed wound healing, whereby it has a higher risk of amputation in poorly controlled diabetic patient with diabetic foot ulcers. Porcine placenta extract (PPE) is a natural waste product that comprises a plenty of bioactive agents including growth factors and antioxidants. It was reported as an effective compound that prevents ROS generation. The goal of this study was to investigate the *in vitro* stimulatory effect of PPE on HG-induced angiogenesis disturbance. The primary endothelial cells (HUVECs) and endothelial cell line (EA.hy926) were treated with HG in the presence of PPE. The endothelial cells (ECs) viability, intracellular ROS generation, migration, angiogenesis, and apoptosis were determined by MTT assay, DCFDA reagent, wound healing assay, tube formation assay, and Annexin V/PI staining, respectively. Additionally, the molecular mechanism of PPE on HG-induced angiogenesis disturbance was investigated by Western blot analysis. The angiogenic growth factor secretion was also investigated by the sandwich ELISA technique. The results showed HG in the presence of PPE significantly decreased intracellular ROS overproduction compared to HG alone. HG in the presence of PPE significantly increased ECs viability, migration, and

angiogenesis compared to HG alone by showing the recovery of PI3K/Akt/ERK1/2 activation. HG in the presence of PPE also decreased ECs apoptosis compared to HG alone by decreasing p53, Bax, cleaved caspase 9, cleaved caspase 3 levels and increasing Bcl 2 level. In summary, PPE attenuated HG-induced intracellular ROS overproduction and improved ECs viability, proliferation, migration, and angiogenesis by showing the recovery of PI3K/Akt/ERK1/2 activation and inhibition of ECs apoptosis. This study suggests PPE ameliorated HG-induced ROS-mediated angiogenesis impairment, whereby it potentially provides an alternative treatment for diabetic wounds.



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ABBREVIATIONS

ABTS	=	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ADP	=	Adenosine diphosphate
AGEs	=	Advanced glycation end products
Akt	=	Protein kinase B
APCs	=	Antigen presenting cells
AR	=	Aldose reductase
ATCC	=	American Type Culture Collection
ATP	=	Adenosine triphosphate
Bax	=	Bcl-2-associated X protein
Bcl 2	=	B-cell lymphoma 2
bFGF	=	Basic fibroblast growth factor
BSA	=	Bovine serum albumin
CAD	=	Coronary artery disease
CD31	=	Cluster of differentiation 31
COX2	=	Cyclooxygenase-2
DAG	=	Diacylglycerol
DCFDA	=	2',7'-dichlorodihydrofluorescein diacetate
DFU	=	Diabetic foot ulcer
DM	=	Diabetes mellitus
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
EA.hy926	=	The human umbilical vein cell line
ECs	=	Endothelial cells
EDTA	=	Ethylenediaminetetraacetic acid
EGF	=	Epidermal growth factor
ELISA	=	Enzyme-linked immunosorbent assay
EPCs	=	Endothelial progenitor cells
ERK	=	Extracellular signal-regulated kinase
$FADH_2$	=	Flavin adenine dinucleotide

ABBREVIATIONS (CONT.)

FBS	=	Fasting blood sugar
FBS	=	Fetal bovine serum
F6P	=	Fructose-6-phosphate
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	=	Glucose transporter
GO	=	Glyoxal
GSH	=	Glutathione
GSSG	=	Glutathione disulfide
G3P	=	Glyceraldehyde 3-phosphate
G6P	=	Glucose 6-phosphate
HG	=	High glucose
HUVECs	=	Human umbilical vein endothelial cells
H_2O_2	=	Hydrogen peroxide
IKK	=	IkB kinases
INS	=	Insulin gene
JNK	=	c-Jun N-terminal kinases
LC MS/MS	=	Liquid Chromatography with tandem mass spectrometry
MGO	=	Methylglyoxal
mg/dL	=	Milligram per deciliter
mg/mL	=	Milligram per milliliter
mL	=	Milliliter
mmol/L	=	Millimoles per liter
MnSOD	=	Manganese superoxide dismutase
MOMP	=	Mitochondrial outer membrane permeabilization
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	=	N-acetylcysteine
NADH	=	Nicotinamide adenine dinucleotide
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NF-kB	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	=	Normal glucose

ABBREVIATIONS (CONT.)

nm	=	nanometer
NO	=	Nitric oxide
NOX	=	NADPH oxidase
NP-40	=	Tergitol-type NP-40 and nonyl phenoxypolyethoxylethanol
PAI-1	=	Plasminogen activator inhibitor-1
PARP	=	Poly (ADP-ribose) polymerase
PI3K	=	Phosphoinositide 3-kinase
PI	=	Propidium Iodide
PKC	=	Protein kinase C
PPE	=	Porcine placenta extract
p38 MAPK	=	p38 mitogen-activated protein kinases
p53	=	Tumor protein P53
ROS	=	Reactive oxygen species
RT	=	Room temperature
rpm	=	Revolutions per minute
SAPK	=	Stress-activated protein kinases
SDH	=	Sorbitol dehydrogenase
SEM	=	Standard error of the mean
TBST	=	1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent
TCA	=	Tricarboxylic acid cycle
TGF	=	Transforming growth factor
UDP	=	Uridine diphosphate
V	=	Volt
VEGF	=	Vascular endothelial growth factor
VEGFR	=	VEGF receptor
WHO	=	World health organization
w/v	=	Weight by volume
µg/mL	=	Microgram per milliliter
µL	=	Microliter
µM	=	Micromolar

CHAPTER I
INTRODUCTION

Background and significance of the study

Hyperglycemia implicates diabetes mellitus (DM). Insulin deficiency and insulin resistance are normally well-known etiology of DM type 1 and 2, respectively, whereby it eventually leads to persistent hyperglycemia in poorly controlled diabetic patients (Campbell, 2007). Persistent hyperglycemia is the pivotal cause of diabetic complications that increases the risk of morbidity and mortality (Bar-Or, *et al.*, 2019; Decker, *et al.*, 2019). The vascular complication in diabetic patients is a major problem that affects systemic organ failure. The excessive production of reactive oxygen species (ROS) has been indicated as a consequence of high glucose (HG)-induced mitochondria dysfunction and HG-induced NADPH oxidase (NOX) overexpression in endothelial cells (ECs) (Rolo, *et al.*, 2006). The ROS overproduction damages ECs which conveys macrovascular and microvascular complications such as coronary artery disease (CAD), peripheral artery disease, nephropathy including angiogenesis disturbance (Fowler, 2008).

Angiogenesis is one pivotal process in wound healing. HG-induced ROS-mediated ECs dysfunction leads to angiogenesis impairment, whereby it eventually impacts to delay wound healing. Despite the molecular mechanism of HG-induced angiogenesis disturbance is still uncleared, the increment of advanced glycation end products (AGEs) and the sorbitol-inositol imbalance that involve with glycation pathway and polyol pathway have been indicated as pivotal factors of angiogenesis disturbance (Kolluru, *et al.*, 2012). Furthermore, HG-induced ROS overproduction directly disrupts DNA, lipids, and proteins, and also involves stress-sensitive intracellular signaling pathways that cause ECs and vascular smooth muscle cell damage (Evans, *et al.*, 2003; Vanessa Fiorentino, *et al.*, 2013; Yuan, *et al.*, 2019). The activation of glycation pathway and polyol pathway has been also indicated as HG-induced endothelial cell senescence, proliferative reduction, and vascular permeable impairment, respectively (Al-Kharashi, 2018; Sawada, *et al.*, 2017). Additionally, HG-induced protein kinase B (Akt) downregulation was also reduced ECs migration and capillary sprouting in angiogenesis, (Ku, *et al.*, 2017) Remarkably, the attenuation

of HG-induced ROS overproduction may improve ECs viable, proliferative, and migrative impairment, whereby it eventually ameliorates angiogenesis disturbance.

Placenta extract has been emphasized in medical research as an alternative treatment for centuries. It derives from several mammal creatures such as bovine, porcine, and human, whereby the placenta expels from the uterus during giving birth. Since the placenta comprises plenty of cytokines, hormones, and growth factors, many researchers and traditional medicine have been focusing the bioactivities on anti-aging therapy and wound repair (Hong, *et al.*, 2010; Tiwary, *et al.*, 2006). In previous studies, porcine placenta extract (PPE) was investigated on the thermal-induced wound in rats. The results demonstrated that PPE reduced wound area through the stimulation of dermal cell proliferation (C. H. Wu, *et al.*, 2003). Moreover, PPE was also attributed as ROS inhibitors, which provided the inhibitory effect of inflammation and oxidation, that attenuated dermatitis by inhibiting ROS generation (Heo, *et al.*, 2018). According to previous studies, this study aimed to investigate the inhibitory effect of PPE on HG-induced intracellular ROS overproduction and the stimulatory effect of PPE on HG-induced ECs dysfunction in terms of viability, migration, pro-angiogenic growth factor secretion, and angiogenesis. PPE could improve HG-induced ROS-mediated ECs viable, proliferative, migrative, and angiogenesis disturbance.

Purposes of the study

1. To evaluate the inhibitory effect of PPE on HG-induced ROS overproduction in ECs
2. To evaluate the stimulatory effect of PPE on HG-induced ECs viable and migrative impairment.
3. To observe the stimulatory effect of PPE on HG-induced angiogenesis impairment.
4. To determine the stimulatory effect of PPE on HG-reduced pro-angiogenic growth factor secretion.
5. To demonstrate the molecular mechanism of PPE on HG-induced endothelial viable, migrative, and angiogenesis disturbance.

Hypotheses of the study

1. PPE could inhibit HG-induced ROS overproduction in ECs.
2. PPE could improve HG-induced ECs viable and migrative impairment.
3. PPE could improve HG-induced angiogenesis impairment.
4. PPE could improve HG-reduced pro-angiogenic growth factor secretion**.**
5. PPE could improve the declination of the HG-induced pro-survival signaling pathway and the increment of HG-induced apoptotic regulatory molecules.

Scope of the study

The investigation of the stimulatory effect of PPE on HG-induced angiogenesis disturbance was performed only *in vitro* model in this study. The derived PPE from Mahidol University had optimized the concentrations for ECs culturing by determining ECs viability. The glucose concentrations were also optimized as HG model by determining ECs viability and intracellular ROS generation. Then, ECs were treated with optimal PPE concentrations in HG. The intracellular ROS generation, ECs viability, ECs migration, angiogenesis, ECs apoptosis, pro-angiogenic growth factor secretion, pro-survival signaling pathway, and apoptotic regulatory molecules were determined and observed in this study by using 2',7' –dichlorofluorescein diacetate (DCFDA) reagent, MTT viability assay, the scratch-wound assay, tube formation assay, the annexin V/PI staining, the sandwich ELISA technique, and Western blot analysis.

Keywords

Porcine placenta extract, high glucose, endothelial cell, diabetes, angiogenesis, wound healing

CHAPTER II
LITERATURE REVIEW

Diabetes mellitus

Diabetes mellitus (DM) is a major metabolic syndrome, whereby it is indicated as global public-health challenges (Alberti, *et al.*, 2005). Since both insulin deficiency and insulin resistance that is defined as diabetic etiology impact on blood sugar uptake, the hyperglycemia is then remarked as DM implication. Normally, the greater or equal to 11.1 mmol/L (200 mg/dL) of random blood sugar has been defined as hyperglycemia according to World Health Organization (WHO). The presence of hyperglycemia can disrupt many systems that convey to diabetes complications, especially cardiovascular malfunction. Long-term hyperglycemia exposure can also promote a high risk of nephropathy, retinopathy, neuropathy, including delayed wound healing (W.H.O., 2019b).

1. Definition of Hyperglycemia

According to the American diabetes association guideline 2019, the definition of hyperglycemia for diabetic patients depends on the diagnostic test of blood sugar level. However, the confirmation of DM onset should be diagnosed at least two tests of blood sampling as follows (W.H.O., 2019a)

Table 1 DM diagnosis tests from blood sampling acquisition (W.H.O., 2019a)

Diagnosis test	Blood sugar level
Fasting blood sugar (FBS)	≥ 126 mg/dL (7.0 mmol/L)
The 2-hour plasma glucose during a 75-g oral glucose tolerance test (2-h PG OGTT)	≥ 200 mg/dL (11.1 mmol/L)
Hemoglobin A1C (HbA1C)	≥ 6.5% (48 mmol/mol)
Random plasma glucose	≥ 200 mg/mL (11.1 mmol/L)

2. DM and hyperglycemia correlation

2.1. The major regulatory hormone of plasma glucose

Hyperglycemia mostly correlates to insulin. Insulin is one of the important hormones that strongly regulate intracellular glucose uptake. Insulin is produced by beta-cell that can be found 50% - 70% in human pancreatic islets (Dolenšek, *et al.*, 2015). Insulin is encoded from the Insulin gene (INS) that locates on chromosome 11p15.5 in the nucleus according to genetic home references. The non-equilibrium of intracellular and extracellular glucose in beta-cell that occurs during plasma glucose elevation can trigger insulin secretion. The glucose metabolism in beta-cell can briefly explain as follows. Commonly, the excessive extracellular glucose diffuses into the beta-cell through glucose transporter 2 (GLUT2). The elevation of intracellular glucose induces glycolysis that yields a high amount of adenosine triphosphate (ATP). Pyruvate is a glycolysis end-product that subsequently translocates into the mitochondria and proceeds in the tricarboxylic acid cycle (TCA). ATP and oxidized nicotinamide adenine dinucleotide (NADH)/ oxidized flavin adenine dinucleotide ($FADH_2$) that is respectively phosphorylated and oxidized are accounted as TCA cycles end-products. The NADH and $FADH_2$ continually donate their electrons to the electron transport chain which comprises various membrane protein complexes lying on mitochondria's inner membrane. The electron donation induces proton (H^+) efflux to mitochondrial intermembrane space and leads to hyperpolarization. The distinct H^+ gradient conveys ATP synthesis through the activation of H^+-induced ATP synthase.

Since the influx of excessive extracellular glucose through GLUT2 leads to the enhancement of intracellular ATP/ADP generation in beta-cell, the ATP overwhelming induces ATP-sensitive potassium (K^+) channel closure, which activates depolarization of the cytosolic membrane. Subsequently, the non-equilibrium of membrane potential evokes voltage-gated calcium (Ca^{2+}) channel opening, which permits Ca^{2+} influx. The intracellular Ca^{2+} elevation then triggers insulin exocytosis, which increases plasma insulin level as shown in Figure 1. The extracellular insulin eventually binds to the insulin receptor at target cells, which induces cellular glucose uptake through GLUT 4 expression on the target cell membrane (Wollheim, *et al.*, 2002).

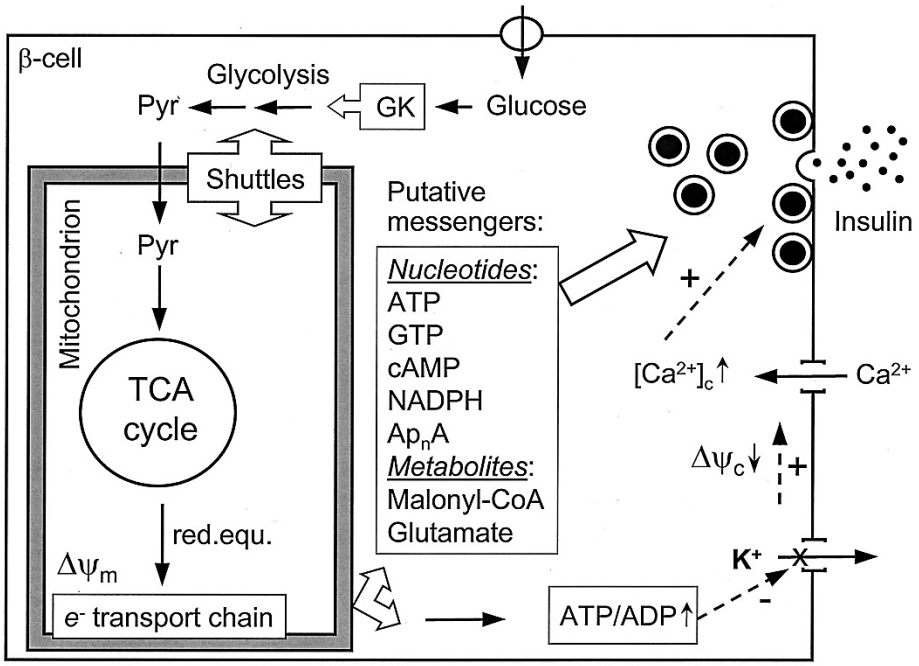

Figure 1 The schematic of plasma glucose elevation-induced insulin secretion by Beta-cell in pancreatic islets. (Wollheim , *et al.*, 2002)

2.2. Insulin secretory defect and DM type I relationship

The insulin secretory defect is commonly a consequence of a beta-cell defect. The destruction of beta-cell by autoimmune has been implied to the cause of DM type I. The cross-presentation of damaged beta-cell to antigen-presenting cells (APCs) initiates beta-cells defect because APCs subsequently recognize and represent beta-cell autologous peptide to adaptive immune cells such as naïve CD4+ T cells (T helpers) and naïve CD8+ T cells (CTLs). These cells are eventually activated to mature T cells which secrete inflammatory cytokines or chemokines against beta-cells. Since beta-cell is the major insulin-secreting cell that has been destructed, insulin production eventually depletes which increases long-lasting plasma glucose elevation. Lately, it leads to persistent hyperglycemia in DM type I (Karlsson, *et al.*, 2000; Yoon, *et al.*, 2005). However, there is another pathophysiology that associates insulin secretory defect. For example, insulin gene mutations may inherently express and lead to inadequate mature insulin production. It has been regarded as the cause of neonatal diabetes (Støy, *et al.*, 2010).

2.3. Insulin resistance and DM type II relationship

DM type II is mainly a consequence of insulin resistance. The glycogen synthesis (glycogenesis) is the major pathway of excessive plasma glucose uptake for energy storage in the skeletal muscle cell and liver tissue. The excessive plasma glucose is up-taken in skeletal muscle cells through insulin-induced GLUT 4 expression on cell membrane during the presence of hyperglycemia and hyperinsulinemia (Shulman, 2000). Normally, the intracellular glucose is converted to Glucose-6-phosphate (G6P) by hexokinase II, which sequentially proceeds the glycolysis pathway. The G6P then raises and induces the excessive glycolysis precursor in case of intracellular glucose elevation. However, there is another regulatory pathway of excessive G6P. Some overwhelmed G6P converts to uracil-diphosphate glucose (UDP-glucose) as a substrate of glucan glycosidic linkage. The glycogenin is a glycogenesis precursor that autocatalyzes UDP-glucose for forming glycosidic linkage. Concomitantly, the glycogen synthase elongates the glycosidic chain along with the cooperation of the glycogen branching enzyme for forming a branch. Finally, mature glycogen is synthesized and stored in muscle and liver tissue. Glycogen will be broken down to G6P that further proceeds in glycolysis, TCA, and ATP synthesis during the existence of inadequate energy.

The induction of persistent hyperglycemia by glycolysis disruption is a cause of DM type II (Shulman, 2000) The glycolysis disruption inherently occurs as a genetic transition by offspring, whereby the expression is a few in long-duration before DM onset. Despite this, the accumulation of free fatty acid also promotes glycogenesis disruption, whereby it affects severe glycogenesis regression (Czech, 2017; Hatting, *et al.*, 2018). Persistent hyperglycemia has been also reported as a cause of the beta-cell defect. Glucose toxicity plays a role resulting in destroying beta-cell in pancreatic islets. High glucose can respectively increase intracellular reactive oxygen species (ROS), oxidative stress, and apoptotic regulatory molecules that are cellular damage factors (Nesher, *et al.*, 1987; Robertson, 2004). ROS-induced beta cell destruction through oxidative stress and apoptosis sequentially impacts the depletion of insulin secretion. The insufficiency of plasma insulin can intensively promote more plasma glucose elevation due to loss of insulin-induced cellular glucose

uptake, whereas glycogenolysis in liver tissue was also triggered due to loss of insulin suppression (Cantley, *et al.*, 2015).

3. Multiple organ failure by persistent hyperglycemic induction

The poorly controlled diabetic patient has a higher risk of multiple organ failure such as neuropathy, retinopathy, and nephropathy (American Diabetes, 2013). Glucose toxicity (Glucotoxicity) is a pivotal factor that impairs several systems through biomolecular regulation. The intracellular ROS is the main mediator that is a consequence of HG-induced mitochondria dysfunction. Additionally, high glucose also affects intracellular ROS overproduction by depriving of redox pathway-related molecules and overexpression of NADPH oxidase (NOX) on the cell membrane (Busik, *et al.*, 2008; Kawahito, *et al.*, 2009). However, ROS production in homeostasis seemingly provides beneficial regulation for cellular signaling transduction. For example, Hydrogen peroxide (H_2O_2) which is one ROS subtype has been implicated as a signaling molecule of thiol oxidation. It involves the cellular signaling process for metabolic adaptation, differentiation, and proliferation (Reczek, *et al.*, 2015). In contrast, ROS overproduction can injure the cell by activating oxidative stress and direct DNA destruction.

4. Molecular mechanism of glucotoxicity

The molecular mechanism of glucotoxicity has correlated and demonstrated in four main hypotheses; increment of polyol pathway activation, enhancement of advanced glycation end-products (AGEs), activation of protein kinase C (PKC), and increment of hexosamine pathway. Despite these four hypotheses, the excessive influx of glucose also correlates with a superoxide overproduction through oxidative phosphorylation in mitochondria (Brownlee, 2001; Campos, 2012). This mechanism has been commonly implied the main cellular damage by glucotoxicity, which directly disrupts DNA, lipids, and proteins.

4.1. Activation of polyol pathway

Polyol pathway is a consequence of the redundant glucose influx that cannot be regulated in regular glucose mechanism, which is glycolysis, TCA, oxidative phosphorylation, and ATP synthesis, respectively. Two important enzymes motivate this pathway; aldose reductase (AR) and sorbitol dehydrogenase (SDH). AR converts intracellular glucose overwhelming to sorbitol along with the $NADPH/NADP^+$ oxidation. Then, the sorbitol is transformed to fructose by SDH along with the NAD^+/ NADH oxidation. The fructose can be phosphorylated to fructose-6-phosphate (F6P) and entered glycolysis pathway by the kinase enzyme reaction. The polyol pathway has been mostly contributed to long-lasting glucose elevation as an alternative pathway in DM (Brownlee, 2001).

Since sorbitol cannot diffuse across the cell membrane, the accumulation of intracellular sorbitol leads to hyperosmolarity and causes osmotic stress. The osmotic stress can subsequently induce cellular damage (Obrosova, *et al.*, 1999). However, there is another mechanism that implicates hyperglycemia-induced cellular damage by the polyol pathway. AR catalyzed NADPH-dependent glucose conversion as the previous mention. NADPH is a fundamental reducing reagent in many metabolisms including glutathione oxidoreduction. Glutathione peroxidase (Active form) is an enzyme that turns H_2O_2 into the water by oxidoreduction. The glutathione sulfhydryl form (GSH) is the intermediary reagent that turns oxidized glutathione peroxidase (Inactive form) to reduced glutathione peroxidase (Active form) again. The glutathione reductase (Active form) is also the second enzyme in this process that turns glutathione disulfide (GSSG) to GSH (Active intermediary reagent). However, the oxidized glutathione reductase (Inactive form) is also turned to reduced glutathione reductase (active form) again for GSSG-to-GSH oxidoreduction by NADPH as shown in Figure 2. Due to the elevation of NADPH-dependent glucose conversion to sorbitol, NADPH may be insufficient for the renewal of glutathione reductase, which leads to depriving GSSG-to-GSH oxidoreduction. Eventually, it leads to inadequate intermediary active form for oxidized peroxidase (inactive H_2O_2 reductase) oxidoreduction and causes H_2O_2 accumulation. (Chung, *et al.*, 2003; Oates, 2002). However, Polyol pathway-induced cellular damage in hyperglycemic

persistency is still uncleared. Moreover, other relevant mechanisms that induce cellular damage such as AGE production and PKC activation are also crucial as relative pathways.

4.2. Enhancement of advanced glycation end-products (AGEs)

Advanced glycation end products (AGEs) are the glycated form of protein or lipid. The sugar (glucose, fructose, galactose) can covalently adhere to non-carbohydrate molecules through the Maillard reaction, which eventually generates AGEs. The exogenous AGE formation rate is slower than the endogenous formation rate because intracellular non-carbohydrate molecules easily interact with G6P and fructose. Because of the elevation of intracellular glucose, the glucose has been broken down through glycolysis and polyol pathway which increases G6P and fructose production. The glucose-derived molecules are subsequently rearranged and attached with intracellular amino acid groups that eventually form AGE precursors [methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone(3-DG)]. MGO, GO, and 3-DG -derived AGEs can damage cells through three major mechanisms; Intracellular protein modification, Extracellular matrix component modification, and plasma protein modification (Singh, *et al.*, 2001).

The modification of AGEs to intracellular protein leads to cellular damage. For example, AGEs induce overexpression of angiopoietin-2 in Müller glia (retina-related glial cell) that causes pericyte loss and capillary regression (Brownlee, 2001; Pfister, *et al.*, 2010). AGEs can also modify extracellular matrix components such as cell-binding domains of type IV collagen that impairs endothelial cell adhesion (Haitoglou, *et al.*, 1992). Nonetheless, AGEs can also modify plasma protein. For example, AGE-induced hypoalbuminemia was found in diabetic mice. Meanwhile, the elevation of plasma AGEs was also found as the relevant factor. Hypoalbuminemia can induce low oncotic pressure that leads to edema (Bhonsle, *et al.*, 2012).

The expression of receptor of AGEs (RAGE) has been also found in many tissues and cell types including endothelial cells, vascular smooth muscle cells, and macrophages. The activation of RAGE triggers downstream signaling (e.g. RAS,

MAPK/ERK, NF-kB) that also influences ROS overproduction (Forrester, *et al.*, 2018; Ott, *et al.*, 2014).

4.3. Activation of protein kinase C (PKC)

Protein kinase C (PKC) is one subtype of the protein kinase family. It composes at least eleven isoforms, of which nine isoforms have been activated by a secondary messenger of lipid signaling. It has been also known as diglyceride or diacylglycerol (DAG). Excessive intracellular glucose-derived products increase the amount of DAG, which enhances PKC activation. The enhancement of PKC activation has been reported as a cause of many systemic abnormalities (Rolo , *et al.*, 2006). For example, PKC induces irregular retinal and renal blood flow by depriving nitric oxide production (NO) and/or endothelin-1 activity (Brownlee, 2001).

4.4. Increment of the hexosamine pathway

The hexosamine pathway implicates glycolysis as one part of fructose-6-phosphate (F6P) degradation. F6P is converted to glucosamine-6-phosphate and UDP-*N*-acetylglucosamine (UDPGlcNAc), respectively. UDPGlcNAc can induce the alteration of Plasminogen activator inhibitor-1 (PAI-1) gene transcription. The hyperglycemia-induced overexpression of the PAI-1 gene promoter has been found in vascular smooth muscle cells. It eventually leads to the elevation of PAI-1 gene transcription. The increment of PAI-1 gene transcription enhances thrombosis and atherosclerosis risk factors and also relates to angiogenesis disruption (Brownlee, 2001).

Even though there are various glucotoxicity mechanisms, the common hyperglycemia-induced cellular damage is superoxide overproduction. Many studies have demonstrated hyperglycemia-induced oxidative stress activation that causes various diabetic complications such as cardiovascular disease, neuropathy, and delayed wound healing (Kolluru , *et al.*, 2012; Pop-Busui, *et al.*, 2006; Vanessa Fiorentino , *et al.*, 2013).

5. Hyperglycemia-induced oxidative stress

Glucose is a major ATP precursor which is necessary for cellular metabolism. The excessive glucose influx implicates the fluctuation of molecular mechanisms. Normally, NADH and $FADH_2$ are glycolysis and TCA cycle end products that play a role as electron donors in oxidative phosphorylation for ATP synthesis. Since ATP synthase is the integrated protein complex that requires hydrogen ion (H^+) influx converting ADP to ATP (energy form), the mitochondrial transmembrane potential is then necessary for the activation of H^+ gradient-dependent ATP synthase. NADH and $FADH_2$ donate their electrons to protein complex recipients that embed on the inner membrane of mitochondria. These recipient complexes are also called electron transport chains. The electron transport chain composed of four protein enzyme complexes, three of four complexes can pump matrix H^+ out to mitochondria intermembrane space by activating sequential electron translocation across these four complexes. Once hyperglycemia existed, NADH and $FADH_2$ which are glucose-derived end products proportionally increase in cytosol and mitochondria. The overwhelmed matrix H^+ efflux subsequently arises and exceeds the threshold of membrane potential. The electron-dependent H^+ pumps (III and IV) are then blocked. The lifetime of electron transportation-intermediated protein is also expended. Electron is then donated to free oxygen molecules instead of the intermediated protein, whereby it leads to plenty superoxide ($\bullet O_2^-$) production (Munusamy, *et al.*, 2009) and affects cellular damage. The mechanism of hyperglycemia-induced ROS overproduction in mitochondria is shown in Figure 2.

Manganese superoxide dismutase (MnSOD) has been indicated as an oxidative phosphorylation-involved superoxide preventer. MnSOD locates in mitochondria that account as one superoxide scavenger (Kanwar, *et al.*, 2007). In the presence of hyperglycemia-induced superoxide overproduction, the low expression of MnSOD is found with an unclear mechanism. Nonetheless, the hyperglycemia-induced anti-oxidase gene disruption has been implicated as the cause of MnSOD low expression. This gene disruption has been induced as a consequence of oxidative stress, which directly damages DNA (Pourvali, *et al.*, 2016). In addition to superoxide overproduction-induced cellular damage, superoxide has also affected hexosamine

activation and glycosylated production (AGEs) (X. L. Du, *et al.*, 2000). Additionally, the overexpression of poly (ADP-ribose) polymerase (PARP) significantly increased in diabetic mice. The PARP overexpression has been indicated as the consequence of hyperglycemia-induced superoxide overproduction-mediated DNA destruction. Superoxide overproduction induces the exposure of DNA strands. PARP is a DNA-repairing enzyme that catalyzes DNA fragment-derived nicotinamide adenine dinucleotide (NAD^+) as two components; ADP-ribose unit and nicotinamide mononucleotide (NMN). ADP-ribose has been repaired and formed a new polynucleotide. However, the PARP overexpression also interrupts free-NAD^+ that influences NADH depletion. Since NAD is a GAPDH substrate in glycolysis, The NAD decrement may also induce glycolysis downstream blockage. Therefore, superoxide namely disrupts GAPDH, whereby it indirectly inhibits glycolysis downstream mechanism. The glycolysis upstream products and precursor (e.g. glucose, G6P, F6P, Glyceraldehyde-6-p) then continue to alternative pathways; polyol, hexosamine, AGE formation, PKC (X. Du, *et al.*, 2003), The correlation between superoxide overproduction and glucotoxicity mechanisms can briefly be illustrated in Figure 3.

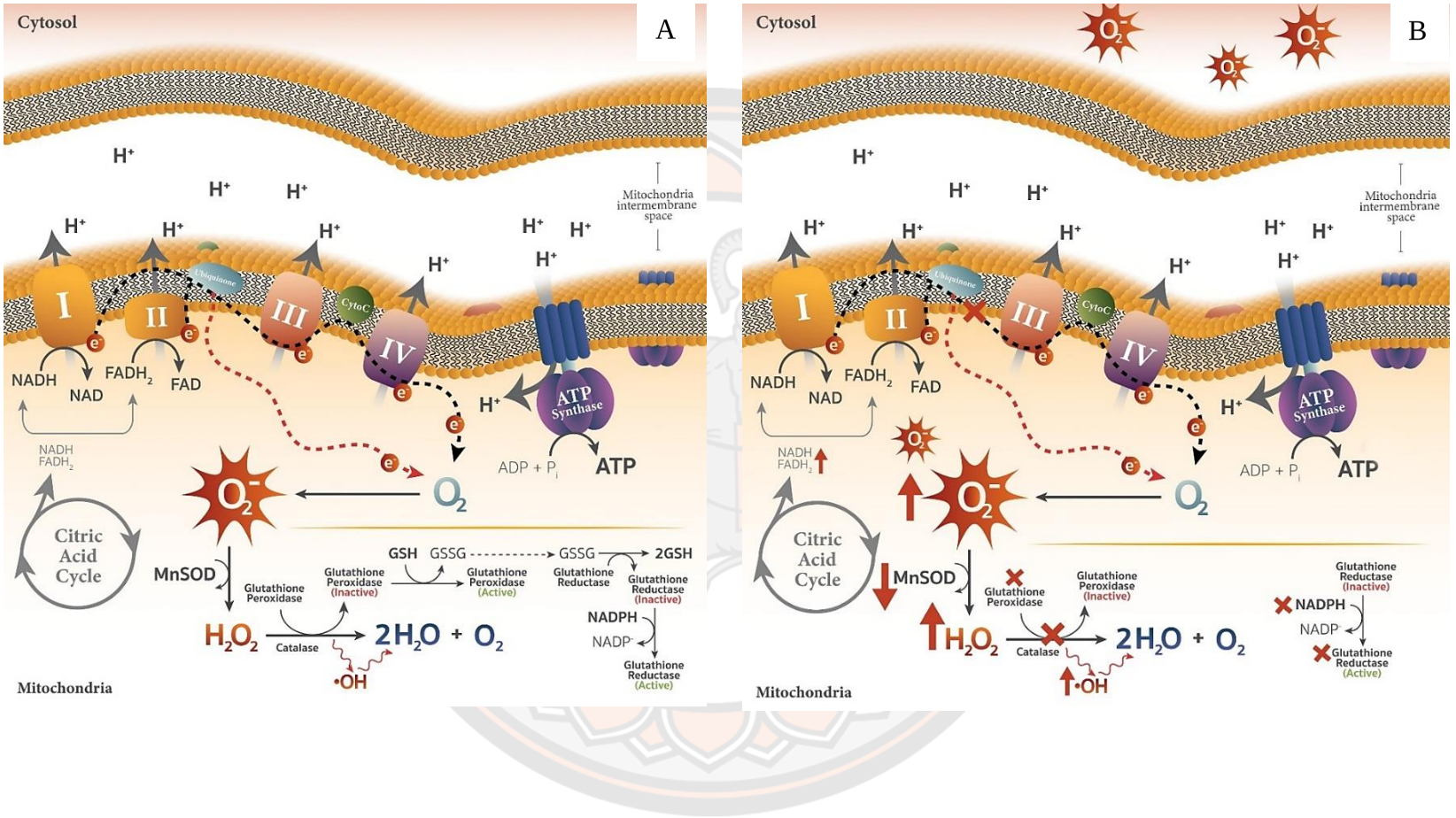

Figure 2 The electron transport chain (ETC)-induced superoxide production in mitochondria.

(A) Oxidative phosphorylation in normal condition. **(B)** Oxidative phosphorylation in high glucose. (Munusamy , *et al.*, 2009)

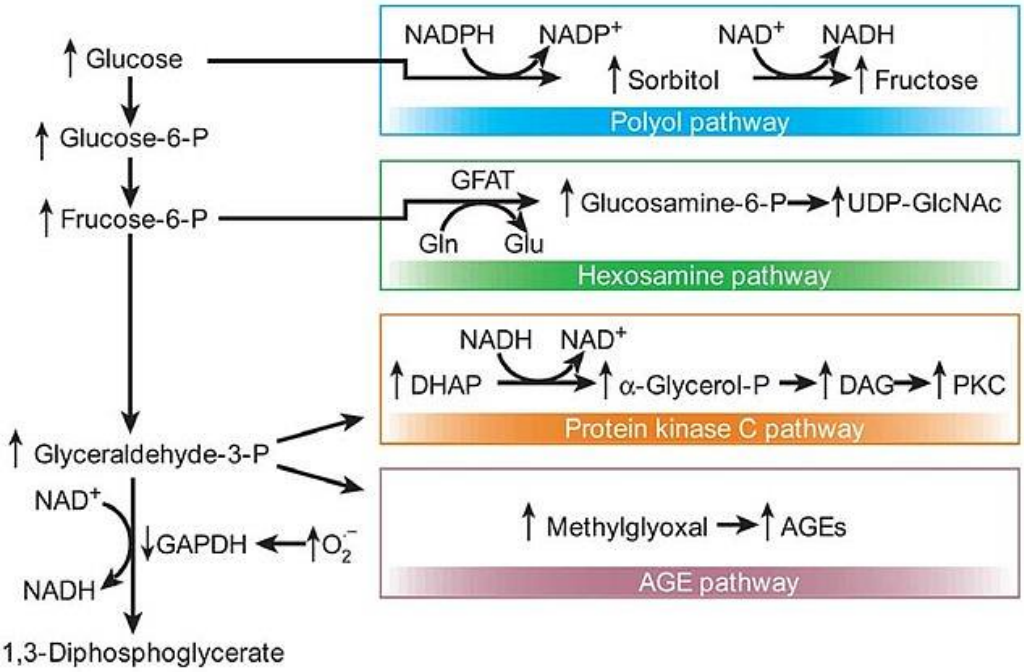

Figure 3 The correlation between glucotoxicity mechanisms and superoxide over-production (Brownlee, 2001).

6. Hyperglycemia-induced stress-sensitive signaling pathway

In addition to hyperglycemia-induced oxidative stress, which directly compromises DNA, lipids, and protein. Stress-sensitive signaling pathways have been also implying as other relevant mechanisms of oxidative stress such as NF-κB, p38MAPK, JNK/SAPK (Evans, *et al.*, 2002; Evans , *et al.*, 2003).

6.1. Oxidative stress-induced NF-κB activation

The NF-κB pathway can be stimulated by various cellular mechanisms. DM is one disease that expresses the elevation of plasma glucose, free fatty acid, and ROS. These factors directly stimulate the NF-kB signaling pathway. NF-κB plays a critical role as an inflammatory and apoptotic mediator. Normally, NF-κB attributes inactive heterodimer in the cytoplasm, which consists of two subunits; p50 and p56. IκB is the protein inhibitor that suppresses these two subunits into inactive forms. Once hyperglycemia-induced ROS overproduction, IκB kinase (IKK) is activated by serine phosphorylation. Active IKK sequentially phosphorylates IκB to a degradative form and leads to NF-κB activation (Evans , *et al.*, 2002). Hyperglycemia-induced NF-κB activation has been indicated as the cause of ECs dysfunction. The overexpression of the COX2 gene that is a cause of inflammatory was dependently found in ECs (Sheu, *et al.*, 2008).

6.2. Oxidative stress-induced JNK/SAPK and p38 MAPK activation

c-Jun N-terminal Kinase (JNK)/Stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (MAPK) also correlate to hyperglycemia. These two signaling pathways can be stimulated by exogenous and endogenous stimuli including the elevation of plasma glucose, free fatty acid, and ROS. The activation of JNK/SAPK induces the transcription factor *cJun*, whereby it sequentially enhances the overexpression of AP-1 recognition site-related genes. These genes have been elucidated as a regulation of redox pathway-involved transcription factors. The NF-κB transcription factor also relates to the AP-1 recognition site, whereby the activation of JNK/SAPK may upregulate the NF-κB pathway. JNK pathway also plays a pivotal role as apoptotic mediator, whereby the presence of JNK inhibitor

resulting in cellular survival has been remarked. The p388 MAPK also plays a role as an apoptotic mediator. The activation of p38 MAPK can stimulate other signaling pathways. For example, the activation of p38 MAPK induces NF-κB activation, which leads to diabetic complications in target organs. The activation of p38 MAPK also disrupts cytoskeleton rearrangement that causes cellular damage (Evans , *et al.*, 2002).

6.3. Oxidative stress-induced PI3K and Akt downregulation

The PI3K/Akt pathway plays a crucial role in cellular metabolism as intermediary transduction such as growth factor signal transduction, cellular survival, protein synthesis, and glucose homeostasis. The insulin receptor involves PI3K/Akt pathway that attributes downstream signaling transduction. The PI3K/Akt pathway plays many roles in hyperglycemic conditions both up- and downregulation such as glucose transporter 4 (GLUT4) activation, Glycogenolysis, Gluconeogenesis, etc. (Huang, *et al.*, 2018). PI3K/Akt also involves a stress-sensitive signaling pathway. It can be accounted as a stress-regulating signaling pathway because IKK is the IκB phosphorylated enzyme that belongs to the downstream regulation of PI3K/Akt, whereby the PI3K/Akt activation normally inhibits active IKK. In the case of PI3K/Akt disturbance or interruption by hyperglycemia, the suppression of active IKK may deprive and subsequently stimulate IκB degradation, whereby it leads to NF-κB activation and induces cellular apoptosis (Brasnyó, *et al.*, 2011; Huang , *et al.*, 2018; Varma, *et al.*, 2005). Interestingly, the mechanistic target of rapamycin (mTOR) and tuberous sclerosis complex 2 (TSC2) have been indicated as upstream transductions of NF-κB and downstream transduction of Akt in phosphatase and tensin homolog (PTEN) deficient in prostate cancer (Dan, *et al.*, 2008). However, there is no evidence of mTOR and TSC2-induced PI3K/Akt-regulated NF-kB activation resulting in diabetic complications, Nevertheless, PI3K/Akt disturbance has been strongly indicated as a consequence of PKC elevation, ROS overproduction, and inflammation that affect insulin resistance in brain tissue cells (Benoit, *et al.*, 2009; Yue, *et al.*, 2012)

Consequently, the hyperglycemia-induced cellular damage may attribute the correlation between four glucotoxicity mechanisms, intracellular ROS overproduction

and stress-sensitive signaling pathways, whereby it regulates molecular mechanisms that causes the systemic failure and diabetic complications (Brownlee, 2001; X. L. Du , *et al.*, 2000; Quijano, *et al.*, 2007)(Figure 4).

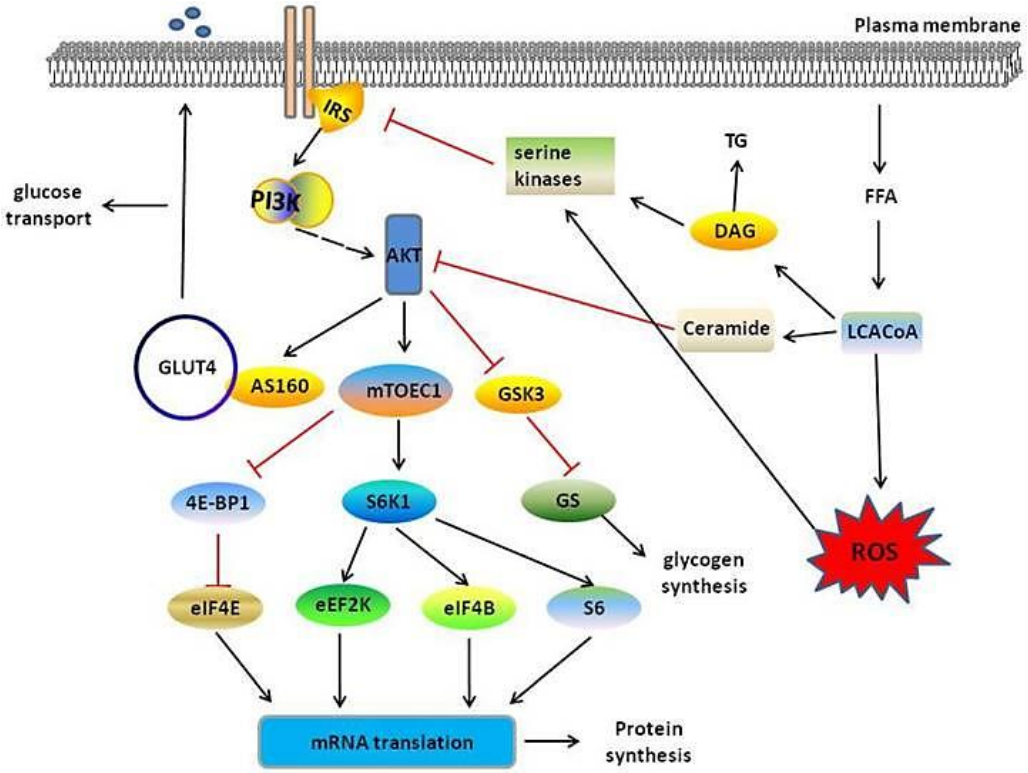

Figure 4 The schematic of ROS-induced PI3K and Akt downregulation in diabetes mellitus type 2 (Huang , *et al.*, 2018).

7. Diabetic complications

As above mentioned, persistent hyperglycemia influences cellular dysfunction through glucotoxicity mechanisms and intracellular ROS overproduction, whereas there are also many non-consensus molecular mechanisms of hyperglycemia-induced cellular damage. Glucotoxicity and ROS overproduction have been strongly emphasized as the cause of diabetic complications. Macro- and micro-vascular complications have been also emphasized as the pathophysiological events that induce diabetic retinopathy, diabetic neuropathy, diabetic nephropathy, and cardiovascular disease in the diabetic patient, etc. (Fowler, 2008) The vascular impairment has been initiated by the hyperglycemia-induced polyol pathway, AGEs formation, hexosamine

pathway, and PKC activation depending on target organs. Oxidative stress has also been implicated as a mediatory linkage of these four pathways. It is also ascribed as a cause of worsening prognosis in poorly controlled diabetic patients (Robertson, *et al.*, 2004). Additionally, the presence of delayed wound healing in diabetic foot ulcers increases the risk of limb amputation, which has been also reported as a consequence of glucotoxicity and oxidative stress (Pecoraro, *et al.*, 1990). Hyperglycemia-induced ROS-mediated angiogenesis impairment has been notified as a cause of delayed wound healing due to the loss of sufficient nutrients and oxygen for granulation tissue formation. However, many wound-related factors are compromised by persistent hyperglycemia such as growth factor depletion, the impairment of cellular migration, proliferation, and viability, and also an imbalance of extracellular matrix accumulation (H. Brem, *et al.*, 2007).

8. Hyperglycemia-induced endothelial dysfunction

Endothelial cell (ECs) apoptosis is a consequence of hyperglycemia exposure, whereby high glucose (HG)-induced ROS overproduction has been implicated as a cause of ECs apoptosis. HG-induced ROS overproduction also impairs physiological ECs functions such as vascular tone and hemostasis. Vasodilation and vasoconstriction are generally the physiological regulation of blood vessels through the activation of specific activators, for example, angiotensin II-induced vascular smooth muscle cell contraction leads the lumen constriction (vasoconstriction), nitric oxide that releases from ECs induces vascular smooth muscle cell relaxation (vasodilation). The hyperglycemia exposure also debases nitric oxide synthesis and provokes vascular tone imbalance. HG-induced atherosclerosis has also been implicated as a consequence of ECs damage by ROS overproduction (Avogaro, *et al.*, 2011). Additionally, hyperglycemia exposure of ECs also impacts pro-angiogenic growth factor secretion, whereby it conducts angiogenesis disturbance and leads to delayed wound healing.

Diabetic foot ulcer and delayed wound healing

Diabetic foot ulcer (DFU) is normally found in diabetic type II patients rather than diabetic type I. The prevalence of global DFU is approximately 5.5% in 2017

and found in males rather than females. The presence of aging, lower body mass index, poorly controlled hypertension, retinopathy, smoking history have been relatively found in almost all diabetic foot ulcer patients (P. Zhang, *et al.*, 2017). DFU has been defined as one pivotal problem in diabetic patients, whereby 33.4% of diabetic patients with DFU suffer from amputation (Lin, *et al.*, 2019). DFU also enhances mortality and morbidity rate in diabetic patients every year (Saluja, *et al.*). The etiology of diabetic foot ulcers has implicated many pathophysiological mechanisms both intrinsic and extrinsic factors. Charcot arthropathy that attributes foot shape abnormality has been elucidated as the intrinsic factor of DFU. The HG-induced peripheral vascular damage is the origin of Charcot arthropathy. The poorly blood perfusion resulting in inadequate nutrients and oxygen supply to extremities. The lack of sufficient nutrients and oxygen leads to necrosis or apoptosis of extremities' tissue. The poorly blood perfusion also induces the disintegration of bone and joints, whereby it provokes foot shape abnormality. Additionally, the HG-induced neuropathy also evokes the worsening progression of DFU. Namely, Charcot arthropathy in diabetic patients increases the risk of calluses, whereby loss of peripheral sensitivity enhances the chance of DFU (Rogers, *et al.*, 2011; Sohn, *et al.*, 2010). Nonetheless, diabetic patients also have a risk of delayed wound healing that promotes a worsening prognosis of DFU, which increases the potential of lower-extremity amputation (Hunt, 2011). Many mechanisms involve delayed wound healing, especially HG-induced molecular mechanism malfunction.

1. Wound healing in the diabetic patient

Wound healing is a sophisticated process that correlates many systems such as coagulation, immune response, bone marrow-derived progenitor cell. This process also involves many cytokines, chemokines, growth factors, and other factors. The wound healing process is classified into four phases; Bleeding (Coagulation), Inflammation, Proliferation, and Remodeling. The impairment of these processes provokes delayed wound healing (Guo, *et al.*, 2010). The four phases and major cellular-mediated wound healing is illustrated in Figure 5 and Table 2.

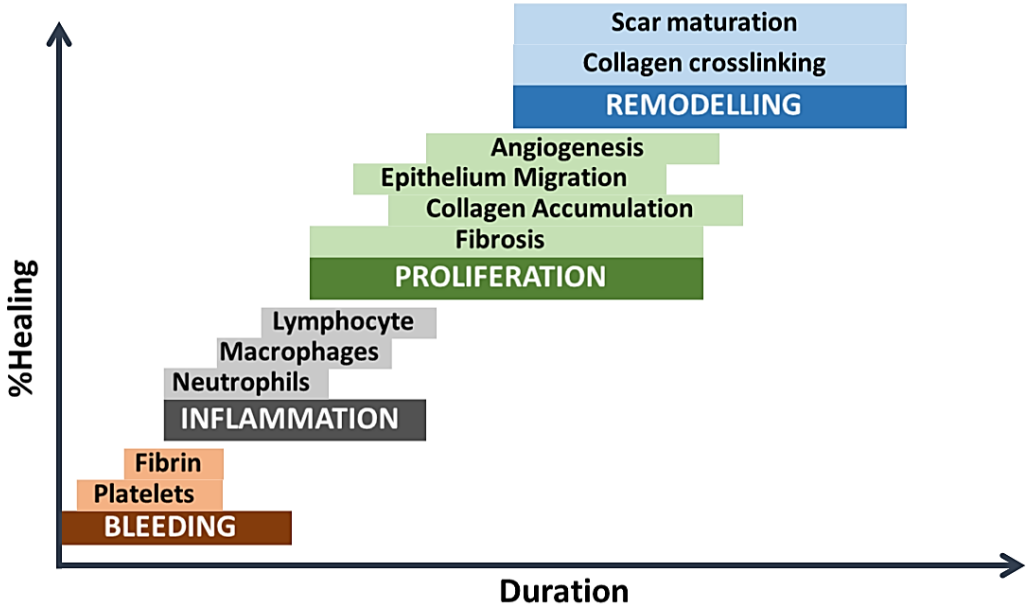

Figure 5 The healing phase in normal wound healing (Guo , *et al.*, 2010)

Table 2 The major events in wound healing process (Brem , *et al.*, 2007)

Healing phases	**Cellular and bio-physiologic events**
Bleeding	Vascular constriction
(Coagulation)	Platelet aggregation and fibrin formation
Inflammation	Neutrophil infiltration
	Monocyte infiltration
	Monocyte-differentiated macrophage
	Lymphocyte infiltration
Proliferation	Keratinocyte proliferation and migration (Re-epithelialization)
	Endothelial migration and proliferation (Angiogenesis)
	Fibroblast proliferation, migration, and differentiation (Collagen synthesis)
	Extracellular matrix formation
Remodeling	Collagen remodeling (Scar degradation)
	Vascular maturation and regression (capillary deformation)

2. HG-impaired cellular migration and proliferation

Many healing factors have been disturbed by hyperglycemia, for example, HG-impaired growth factor secretion, HG-induced angiogenesis disturbance, macrophage malfunction, and the impairment of dermal-related cell proliferation and migration (Harold Brem, *et al.*, 2007). The HG-impaired cellular migration and proliferation have been indicated as a consequence of intracellular ROS overproduction and glucotoxicity. Re-epithelialization is one pivotal process in the proliferative phase that shallows the wound bed (wound depth). This process is mediated by hypoxia-inducible factor 1-alpha (HIF-α), which induces keratinocyte proliferation and migration. The hypoxia also induces the differentiation of basal layer cells to keratinocytes (O'Toole, *et al.*, 1997). Even keratinocytes can proliferate and migrate by hypoxic triggering, the granulation tissue formation also requires sufficient blood supply to convey complete wound repairing. Since HG-induced ROS overproduction and glucotoxicity provokes ECs dysfunction, especially in terms of proliferative and migrative impairment, which then impair ECs sprouting and angiogenesis (Kolluru , *et al.*, 2012).

3. HG-induced angiogenesis disturbance

Angiogenesis is an important process for providing a new capillary in the target tissue. The new capillary can provide adequate growth factors and oxygen for granulation tissue formation (Frank, *et al.*, 1995). New capillaries in wound sites also provide nutrients, oxygen, and hormones for keratinocyte and fibroblast proliferation and migration (Liu, *et al.*, 1995). Therefore, angiogenesis plays a key role in healing progression.

Since angiogenesis plays a key role in granulation tissue forming and healing progression, the absence of endothelial progenitor cells and pro-angiogenic growth factors have implicated angiogenesis worsening and poorly granulation tissue formation (Avogaro , *et al.*, 2011). However, the pre-existed ECs can proliferate and migrate to form a new capillary by activating pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).

This process is generally called ECs sprouting. In diabetic patients, HG has impaired ECs proliferation, migration, and sprouting (Carmeliet, 2003).

3.1. HG-induced endothelial proliferative impairment

HG-induced PKC activation affects PI3K/Akt downregulation. In a previous study, the presence of Akt downregulation was found in ECs culturing in HG at 20 mM and 40 mM of D-glucose. They found D-glucose 20 mM reduced the phosphorylation of Akt at tyrosine 308 (p-Thr^{308}) rather than serine 473 (p-Ser^{473}), It suggested HG-induced Akt downregulation through Thr^{308} binding site. Furthermore, the Akt downregulation can evoke p53 overexpression and reduce cell viability and proliferation (Varma , *et al.*, 2005).

3.2. HG-induced endothelial migrative impairment

Similarly, the PI3k/Akt pathway has been also illustrated the regulation of ECs migration. The downregulation of PI3K/Akt by HG reduced ECs migration, proliferation, including angiogenesis (Yu, *et al.*, 2006). However, the consensus of insight mechanistic signaling has not been clearly illustrated.

4. HG-induced cellular resistance to pro-angiogenic growth factors

VEGF is a major pro-angiogenic growth factor, whereby it plays a role in ECs sprouting in angiogenesis for wound healing, tumor growth, and embryonic development. (van Hinsbergh, *et al.*, 2008). There are various subtypes of VEGF receptors (VEGFR) depending on the target organ. Normally, VEGF-A binds to VEGFR-1 and VEGFR-2. It plays a crucial role in pathophysiological angiogenesis such as wound repair and tumor growth. VEGF-C/D commonly binds to VEGFR-3, which plays a critical role in embryonic development and lymphangiogenesis (Shibuya, 2011).

HG-induced ECs resistance to VEGF has been also implied as a cause of angiogenesis disturbance, whereby the response of VEGF resistance is different depending on the target tissue. There are three possible hypotheses of VEGF resistance by HG. Firstly, the defect of VEGFR expression on the cell membrane. Secondly, the abnormality of VEGF signal transduction. Thirdly, signal generation

deficiency. The abnormality of VEGF signal transduction has been potentially implied as a consequence of hyperglycemia. Since hyperglycemia induces AGEs formation, ROS overproduction, and PI3K/Akt downregulation, these concomitant mechanisms provoke VEGF signaling cascade malfunction. However, the mechanistic insight of HG-induced angiogenesis disturbance is still unclear and demonstrates paradox activation. HG-induced excessive VEGF secretion and VEGFR overexpression is indicated as a cause of angiogenesis overgrowth, whereby it evokes diabetic retinopathy. In contrast, HG-induced cellular resistance to VEGF potentially impairs angiogenesis. Additionally, HG-induced ECs viable, proliferative, and migrative impairment concomitantly evoke angiogenesis worsening, which impacts inadequate blood circulation to the wound site and induces delayed wound healing (Waltenberger, 2009)(Figure 6).

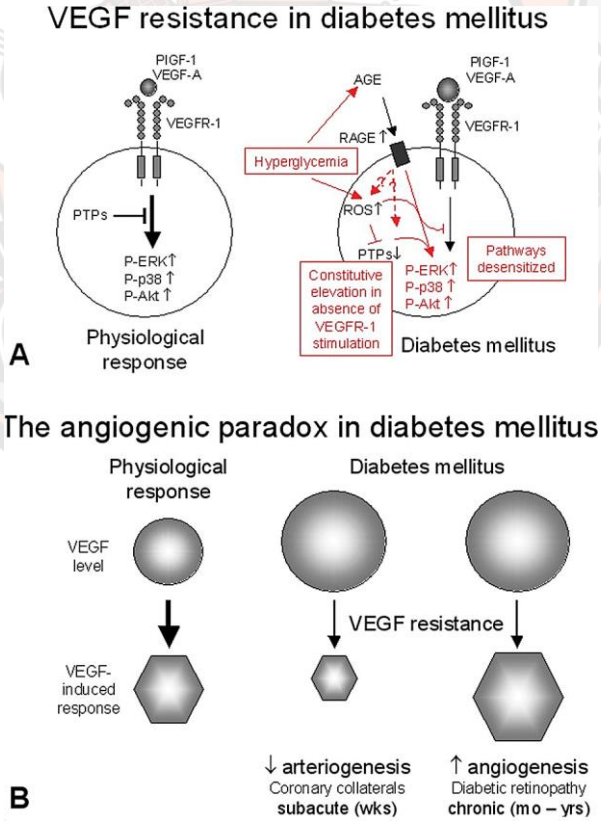

Figure 6 The etiology of hyperglycemia-induced VEGF resistance (A) and paradox mechanism of angiogenesis disturbance (B) (Waltenberger, 2009)

5. Therapeutic procedure and wound care in diabetic patients

There are many therapeutic procedures and wound care in diabetic patients. Metformin has been used for the regulation of hyperglycemia to suppress hepatic glycogenolysis, induce cellular glucose uptake, reduce intestinal glucose absorption (Foretz, *et al.*, 2014). The well-controlled plasma glucose in diabetic patients has been suggested and indicated a lower risk of delayed wound healing and amputation. In case diabetic wound care, there are also many topical drugs or wound managements that accelerate the healing process. For example, nicotine was used to improve angiogenesis that leads to wound healing acceleration through the activation of endothelial nicotinic acetylcholine receptors (nAChRs) (Jacobi, *et al.*, 2002). The topical VEGF was used to enhance angiogenesis that evokes healing improvement through bone marrow-derived endothelial progenitor cells (EPCs) recruitment (Galiano, *et al.*, 2004).

Placenta extract

Placenta extract has been used as an alternative medicine for centuries. It is derived from the placenta which comprises plenty of cytokines, hormones, growth factors, nucleic acid, etc. The placenta consists of the umbilical cord, placenta membrane, and parenchymal, it connects between the maternal uterus and fetus for nutrient and waste transfer in gestation. Additionally, the placenta also acts as an essential growth factor reservoir that provokes fetal development. (Farmer, *et al.*, 1997; Pan, *et al.*, 2017). The component of major cytokines and growth factors in porcine placenta extract is demonstrated in Table 3 and Table 4.

1. Placenta extract preparation

There are two regular preparations of placenta extract Firstly, Aqua-based solvent mostly binds to polar molecules. Aqua-based extract mostly composes of polar molecules (e.g. peptides/proteins, amino acids, nucleotides, carbohydrates, and a few hydrophilic-compartment lipids). In a previous study, the aqua-based solvent was used to extract placenta that obtains plenty of growth factors, whereby it was emphasized for wound healing enhancer (Nath, *et al.*, 2007). Secondly,

hydroalcoholic-based solvent mostly suits for less polar and hydrophobic molecules (e.g. glycosphingolipids cholesterol, triglycerides, lipoprotein, and all hydrophobic substances) In a previous study, the human placenta was extracted by using hydroalcoholic-based solvent, whereby the glycosphingolipids was emphasized as a skin pigmentation activator (Pal, *et al.*, 1995).

Table 3 The major cytokines of porcine placenta extract and their functions in a previous study (Farmer , *et al.*, 1997)

Cytokines	Functions
Interleukin-1 (IL-1)	Regulates immune cells and inflammatory response
Interleukin-2 (IL-2)	Regulates T_{reg}-cell differentiation to against immunity
Interleukin-4 (IL-4)	Activates B-cell and T-cell proliferation, and up-regulate MHC class II production.

Table 4 The major growth factors of porcine placenta extract and their functions in a previous study (Farmer , *et al.*, 1997)

Growth factors	Functions
Granulocyte-colony stimulating factor (G-CSF)	Involves cell cycle regulation and mediates neutrophil progenitor differentiation and proliferation
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Involves many immune cell type especially macrophage and eosinophil proliferation a maturation
Epidermal growth factor (EGF)	Activates cellular proliferation, migration, and survival
Fibroblast growth factor (FGF)	Induces fibroblast and endothelial cell proliferation and migration for tissue regeneration and wound healing
Hepatocyte growth factor (HGF)	Possess a mitogen task for keratinocyte and melanocyte, Provokes hepatocyte production

Table 4 The major growth factors of porcine placenta extract and their functions in a previous study (Farmer , *et al.*, 1997) (Cont.)

Growth factors (Cont.)	Functions (Cont.)
Insulin-like growth factor (IGF)	Involves muscle cells and cells proliferation including regulation of fet0-placenta growth in gestation
Platelet-derived growth factor (PDGF)	Stimulates cell differentiation, proliferation, migration, and cellular survival
Transforming growth factor (TGF)	Promotes epithelial proliferation and differentiation including tissue remodeling
Vascular endothelial growth factor (VEGF)	Regulates angiogenesis and vascular development including hematopoietic and neuro-protection

2. Pharmaceutical effects of placenta extract

Since placenta extract comprises many growth factors for cellular renewal and proliferation, the dermal cosmetic industry has been then emphasizing this effect as a wrinkle removal (Nair, *et al.*, 2002). However, there is another pharmaceutical effect of placenta extract in addition to growth stimulation.

2.1. Antibacterial effect

The aqueous placenta extract has been indicated as an antibacterial agent. It can inhibit the growth of clinically isolated bacteria such as *Escherichia coli*, *Streptococcus aureus* including drug-resistance strains such as *E. coli* DH5α Pet-16 AmpR and *Pseudomonas aeruginosa* CamR. A mixture of polydeoxyribonucleotides in the extract was indicated as the active agent (Datta Chakraborty, *et al.*, 2005). In another study, the placenta membrane (amnion and chorion) extract has been indicated as *Streptococcus pneumonia* and biofilm production inhibitor. They suggested many hydrolases, ribonuclease, protease, and other antimicrobial proteins and peptides that contain in this extract may provide antimicrobial property (Yadav, *et al.*, 2017).

2.2. Anti-inflammatory effect

The placenta extract also has anti-inflammatory effects and analgesic effects. It significantly inhibited nitric oxide, tumor necrosis factor-α, and cyclooxygenase-2 expression, which are pro-inflammatory mediators. It also increased the pain threshold and decreased rat paw swelling (Lee, *et al.*, 2011). However, the placenta extract did not has a beneficial effect for rheumatoid arthritis treatment in mice (Park, *et al.*, 2012). Therefore, the mechanistic insight of the anti-inflammatory effect of placenta extract is still unclear.

2.3. Anti-oxidative effect

The placenta extract also has an anti-oxidative effect. In a previous study, placenta extract decreased nitric oxide overproduction that damage hemoglobin in the erythrocyte (S. Rozanova, *et al.*, 2012). Many researchers attend to clarify and purify the antioxidant substances in human placenta extract. In a previous study, uracil, tyrosine, and phenylalanine have been indicated as the major structure of antioxidant substances in placenta extract (Togashi, Takahashi, Kubo, *et al.*, 2000). They further investigated these purified substances on ethanol-induced oxidative hepatic tissue. The results elucidated the placenta extract activated glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, and superoxide dismutase, whereby it suppressed oxidative stress. However, crude placenta extract also has another anti-oxidant such as thiobarbituric acid reaction substance (TBARS), which is unable to observe in purified extract (Togashi, Takahashi, Watanabe, *et al.*, 2000). In another study, L-tryptophan has been indicated as the main placenta-derived antioxidant, which affects lipid peroxidative suppression (Watanabe, *et al.*, 2002). Thus, placenta extract can be stated as an antioxidant reservoir. Even placenta potentially comprises various antioxidative substances, the freshly prepared extract, and well preservative extract should be concerned to preserve the antioxidant properties. In a previous study, the rapid freezing placenta extract preserved higher antioxidant activity than slow-freezing (-20^{o}C) and non-freezing after thawing, respectively.(S. L. Rozanova, *et al.*, 2010).

2.4. The stimulatory effect on wound healing

placenta extract contains abundant growth factors and cytokines that impact to wound process in terms of the activation of proliferation and migration. In the previous study, placenta extract accelerated wound closure through the regulation of TGF activation in an early phase and VEGF activation in the late phase (Hong , *et al.*, 2010). The VEGF activation involves in the angiogenesis process in wound healing. The amniochorion in the placenta has been identified as the accumulative source of pro-angiogenic growth factors, whereby it eventually provides a high amount of these growth factors in the extract (BURGOS, 1986). The plenty of pro-angiogenic growth factors in the extract has also been illustrated as the major activation of ECs-derived protease plasminogen activator and collagenase increment, which is important for ECs growth and mobility (Presta, *et al.*, 1985).

3. Porcine placenta extract in current research

Porcine placenta extract (PPE) is an animal-derived compound. It is a natural wasted product in the agricultural industry, which obtain easier than the human placenta. However, the analogous mammal-derived placenta extract provides less safety than autologous-derived extract for human utilization. (Mitsui, *et al.*, 2015). Therefore, the safety and toxicology should be clarified before the conceptual project investigation and clinical trial.

Currently, PPE has been investigated its bioactivities on many diseases. For example, PPE was investigated the stimulatory effect on the thermal-induced wound in rats, The results showed PPE decreased wound area in rats by provoking basic fibroblast growth factor (bFGF) and transforming growth factor β1 (TGF-β1) expression (C. H. Wu , *et al.*, 2003). Moreover, PPE has attributed the anti-inflammatory and anti-oxidative agent for dermatitis attenuation by inhibiting ROS generation (Heo , *et al.*, 2018). PPE also expressed melanogenesis suppression in melanoma cells through the regulation of antioxidant enzyme gene overexpression (Yamasaki, *et al.*, 2014).

CHAPTER III
METHODOLOGY

The experimental design was categorized and described in three major parts as follows.

A. The modeling of high glucose (HG) condition and optimization of PPE concentrations by determining ECs viability and intracellular ROS generation.
B. The stimulatory effect of PPE on HG-induced ROS-mediated endothelial cell (ECs) viable, proliferative, migrative, angiogenesis, apoptosis, and pro-angiogenic growth factors secretion impairment by determining ECs viability, migration, tube formation, intracellular ROS generation, and pro-angiogenic growth factors secretion.
C. The molecular mechanism of PPE on HG-induced ROS-mediated angiogenesis impairment by using Western blot.

All experiments were performed by culturing human umbilical vein endothelial cell line (EA.hy926) and/or primary human umbilical vein endothelial cells (HUVECs) with normal glucose (NG) alone, High glucose (HG) alone, and HG in the presence of PPE. HG in the presence of N-acetylcysteine (NAC). NAC treatment was used as a positive control for PPE treatment due to its ability as a ROS scavenger. In part A, the intracellular ROS production and ECs viability were determined for modeling HG condition by culturing EA.hy926 cells with various glucose concentrations. The cell cytotoxicity was also determined to optimize the efficient PPE concentrations by culturing EA.hy926 cells with various PPE concentrations. In part B, The intracellular ROS generation, ECs viability, ECs apoptosis, ECs migration, tube formation, and pro-angiogenic growth factors secretion were determined to investigate the stimulatory effect of PPE on HG-induced angiogenesis disturbance by culturing EA.hy926 cells and/or HUVECs with NG alone, HG alone, HG in the presence of PPE, and HG in the presence of NAC. In part C, the pro-survival signaling pathway and apoptotic regulatory molecules were determined to investigate the molecular mechanism of PPE on HG-induced angiogenesis disturbance by using Western blot analysis.

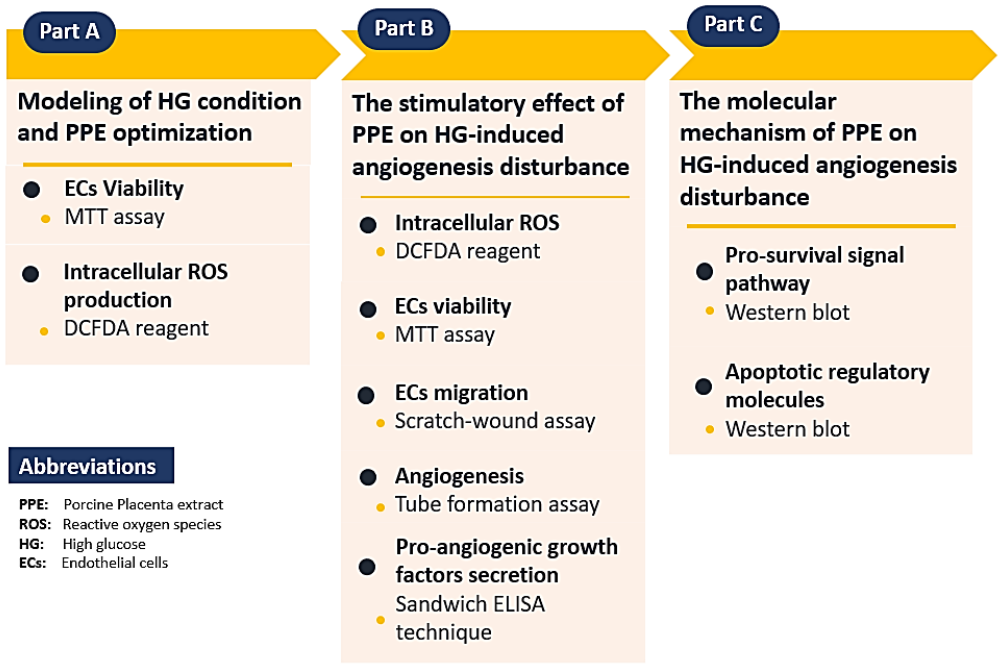

Figure 7 The schematic of the experimental design in this study

In part A, the modeling of HG condition and optimization of PPE concentration was conducted. In part B, the investigations of PPE's stimulatory effect on HG-induced angiogenesis disturbance were performed. In part C, the molecular mechanisms of PPE on HG-induced angiogenesis disturbance were determined.

Cell culture techniques for endothelial cells

1. Endothelial cells

There are two kinds of endothelial cells (ECs) that were used in this study. Firstly, the human umbilical vein endothelial cell line (EA.hy926)(ATCC-CRL2922) was kindly provided as a gift by Assoc. Prof. Sarawut Khumphue, Ph. D., Biomedical Engineering Institute (BMEI), Chiang Mai University, Chiang Mai, Thailand. The primary human umbilical vein endothelial cells (HUVECs)(ATCC PCS-100-013) was purchased from ATCC in the US via a distributor in Thailand (Gibthai Co., Ltd., Samsennok Huay Kwang, Bangkok, Thailand).

EA.hy926 cells are the endothelial cell line that was established by fusing primary human umbilical vein cells with a thioguanine-resistant clone of A549. This

cell has been widely used to represent *in vitro* vascular endothelial cells in many studies, whereby it has been confirmed the presence of endothelial cell characteristics (Ahn, *et al.*, 1995).

HUVEC is a primary endothelial cell that has been widely used to represent *in vitro* primary vascular endothelial cells in several cardiovascular research.

2. Cell culture protocol

EA.hy926 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco®), supplemented with 10% fetal bovine serum (Gibco®) according to the recommended protocol. The antibiotic/antimycotic was also added for preventing contamination. This cell line was maintained in a T-75 cultured flask and incubated in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C until 80% confluence. HUVECs (ATCC®) were cultured in vascular basal medium supplemented with endothelial cell growth kit-BBE, and penicillin-streptomycin-amphotericin B solution according to the recommended protocol. This primary cell was maintained as a previous indication. The cultured medium was replaced every 2-3 days. Passages 6-10 of HUVECs and passages 6-15 of EA.hy926 cells were used in all experiments.

3. Subculture protocol

EA.hy926 cells and HUVEs were subcultured according to the recommended protocol. EA.hy926 cells and HUVECs subculture were performed in terms of the presence of 80%-100% confluences. Briefly, the remained medium was discarded and washed with 1X PBS. The cells were subsequently detached by adding 1X 0.25% (w/v) Trypsin/EDTA solution (for EA.hy926 cells) or Trypsin/EDTA for primary endothelial cell (for HUVECs) and incubating at 37ºC for 1-3 minutes or until the presence of rounded shape. Gentle agitation may be performed in case of the presence of tight attachment. The trypsin was neutralized by adding 10% FBS complete medium (for EA,hy926) or Trypsin neutralizer (for HUVECs). The cell suspension was gently aspirated and precipitated at 1500 rpm for 5 minutes. The cell pellet was collected and resuspended to the desired concentration. The cell suspension was

transferred into a T-75 cultured flask and maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C.

4. Cryopreservation protocol

EA.hy926 cells and HUVECs were initially performed as same as the subculture protocol, whereby there is no negligence of any cell suspension. The cell suspension after subculturing was centrifuged at 1500 rpm for 5 minutes. The cell pellet was collected and resuspended with a freezing medium (10% DMSO in FBS). The cell suspension was immediately dispensed into precooled cryovial tube with the desired volume (0.8 – 1 mL). These cryovial tubes were disinfected with 70% alcohol before loading into the cell-freezing container (Bio-gener™). The freezing container was immediately stored at -20^{o}C overnight for cooling down. Subsequently, the freezing cryovial tubes were immediately stored in a -80^{o}C freezer or liquid nitrogen reservoir.

5. Thawing protocol

The freezing cryovial tube was transferred by loading it in the cell freezing container. The freezing cryovial tube was promptly thawed in a water bath at 37^{o}C until the presence of jelly-like matter in the cryovial tube. After disinfection, cells were gently aspired and transferred into a T-75 cultured flask contained with EA.hy926 complete medium or HUVECs complete medium for 6 mL. Subsequently, cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. The cultured medium was replaced with EA.hy926 complete medium or HUVECs complete medium for 6-7 mL in the next day.

6. Cell counting

The cell number was counted during the subculture process and experiment performing. The principle of cell counting bases on trypan blue exclusion assay. The trypan blue infiltrates into the death cell that loses the intact cell membrane. In brief, the cell suspension was dispensed into a 96-well plate for 20 µL. The trypan blue was immediately added at a 1:1 ratio (20 µL). Then, the mixture was wisely mixed and dispensed into a hemocytometer. The viable cell number was counted by excluding a

cell in the presence of trypan blue inside. Subsequently, the viable cells in suspension were calculated according to indicated equation.

$$Cell\ number\ (cells/\mu L) = \frac{number\ of\ cell\ couting\ \times dilution\ factor}{number\ of\ large\ counting\ chamber}$$

The viable cells in total cell suspension can be also calculated by multiplying with the total volume of cell suspension in µL.

Crude porcine placenta extract preparation

The crude PPE was provided by Assoc. Prof. Rutaiwan Tohtong, Ph. D., Faculty of Science, Mahidol University, Bangkok, Thailand. In brief, the porcine placenta that is a natural waste product from farming was cleaned and mechanically homogenized in phosphate buffer saline (PBS) solution. Then, the homogenate was sonicated and centrifuged at 4°C for 1 hour. Then, the supernatant was filtrated with 0.2 µm sterile filters. Our crude PPE was aliquoted with sterile technique and stored at -20 ^{o}C. The freezing PPE was thawed at room temperature and wisely mixed before performing in all experiments.

The model of high glucose condition

The modeling of the HG condition was performed according to Karbach, *et. al.* method (Karbach, *et al.*, 2012). Briefly, EA.hy926 cells were seeded in a 96-well plate (NEST®) at 5 x 10^3 cells/well and incubated overnight. Then, ECs were treated with various glucose concentrations in a 0.5% serum medium. D-glucose (Sigma, US) 5.5 mM (110 mg/dL) represents to normal glucose (control). The intracellular ROS generation was also determined to confirm the stimulatory effect of optimal glucose concentration on ROS generation.

1. Determination of HG-induced endothelial viable impairment

1.1. The principle of MTT viability assay

MTT viability assay is an enzyme-based method by using 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT). This reagent can interact with mitochondria succinate dehydrogenase and oxidoreductase enzymes. These enzymes involve the equivalent of mitochondria, whereby it induces the metabolic active status of the cell. MTT reagent can penetrate the cell membrane and mitochondria. Then, MTT can be reduced to formazan dye (dark purple) by these enzymes. The formazan dye is a non-soluble crystal that can be dissolved by an organic solvent such as DMSO. The cell viability can be quantified by using spectrophotometry with a certain wavelength (500 – 600 nm). The optical density proportionally represents the number of viable cells (active status) (Gerlier, *et al.*, 1986).

1.1. Determination of cell viability by using MTT viability assay

The MTT viability assay was performed according to the previous study (Riss, *et al.*, 2004). Briefly, the cultured medium was discarded before the dispensation of MTT working solution for 100 µL. This MTT working solution consists of MTT reagent (Amresco®) 0.5 mg/mL in a serum-free medium. Then, the cells were incubated in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C for 4 hours. The formazan crystal was observed under inverted microscopy before adding dimethyl sulfoxide (DMSO) (Amresco®) with gentle mixing. The DMSO can dissolve the formazan dye for quantification of cell viability by using a microplate reader (PerkinElmer).

2. Determination of HG-induced intracellular ROS overproduction

2.1. The principle of DCFDA reagent for determining intracellular ROS

Since HG has been reported as a cause of ROS overproduction, the determination of intracellular ROS is then emphasized for optimization of the HG model in this study. The 2'-7'-dichlorodihydrofluorescein diacetate (DCFDA) is a cell-permeable chemiluminescent agent that can be oxidized by ROS. The principle of

DCFDA reagent against ROS production can briefly explain as follows. DCFDA can be deacetylated to non-fluorescent compounds by cellular esterases that arrange on the cell membrane. After penetration, it is subsequently oxidized by intracellular ROS and transformed into a fluorescent agent (DCF). The intracellular ROS can be quantified by using fluorescence spectrophotometry with certain excitation and emission wavelength (495 nm excitation /529 nm emission).

2.2. Determination of intracellular ROS by using DCFDA reagent

The intracellular ROS determination was performed by using DCFDA reagent according to the previous study (Zhu, *et al.*, 2015b). Briefly, After HG treatment at the indicated time, the cultured medium was discarded and washed with 1X PBS twice. DCFDA 25 µM in the serum-free medium was subsequently added to each well for 100 µL. Cells were incubated in a dark humidified atmosphere of 95% air and 5% carbon dioxide at 37°C for 30 minutes. Then, the DCFDA reagent was discarded and replaced with 1X PBS. The fluorescence was immediately determined by using a microplate reader at 485 nm excitation/535 nm emission or inverted microscopy with the FITC filter.

Optimization of PPE concentration

The optimization of PPE concentration was performed by determining ECs viability. In brief, EA.hy926 cells were seeded in a 96 well plate (5 x 10^3 cells/well) and incubated overnight. Then, cells were treated with various PPE concentrations at 0, 5, 10, 20, 40 µg/mL in 0.5% serum medium. The PPE 0 µg/mL represents a control. After treatment, the MTT viability assay was performed according to the previously indicated protocol.

The inhibitory effect of PPE on HG-induced ROS overproduction

The inhibitory effect of PPE on HG-induced ROS overproduction was performed by using a DCFDA reagent. In brief, EA.hy926 cells and HUVECs were seeded in clear bottom black 96-well plate (1 × 10^4 cells/well) and incubated overnight. Then, cells were washed and treated with normal glucose (NG) alone, high

glucose (HG) alone, and HG in the presence of various PPE concentrations. NG in the presence of hydrogen peroxide (H_2O_2) 100 µM was used as a positive control of HG alone. N-acetylcysteine (NAC) is a ROS scavenger. HG in the presence of NAC 15 µg/mL was used as a positive control of HG in the presence of PPE. After treatment, the determination of intracellular ROS was performed by using DCFDA reagent according to the previously indicated protocol.

The stimulatory effect of PPE on HG-induced endothelial viable impairment

The stimulatory effect of PPE on HG-induced ECs viable impairment was performed by determining ECs viability. In brief, EA.hy926 cells and HUVECs were seeded in a 96-well plate (5×10^3 cells/well) and incubated overnight. Then, ECs were washed and treated with normal glucose (NG) alone, high glucose (HG) alone, and HG in the presence of various PPE concentrations. HG in the presence of NAC 15 µg/mL was used as a positive control of HG in the presence of PPE. After treatment, the determination of ECs viability was performed by using the MTT viability assay according to the previously indicated protocol.

The inhibitory effect of PPE on HG-induced endothelial cell apoptosis

To confirm the inhibitory effect of PPE on HG-induced ROS-mediated ECs viable impairment, the inhibitory effect of HG-induced ECs apoptosis was determined by using Annexin V/Propidium Iodide (PI) staining. HUVECs were treated with NG alone, HG alone, HG in the presence of PPE (2.5, 5, 10, 15 µg/mL), and HG in the presence of NAC 15 µg/mL for 24 hours. After treatment, the ECs apoptosis was determined as follows.

1. The principle of cell apoptosis determination by using Annexin V/PI staining

The principle of the annexin V/PI staining method bases on the characteristic of cell apoptosis. There are many cell death subtypes such as apoptosis, necrosis, autophagy, etc. Each subtype attributes a different characteristic of cell death. Annexin V can bind with high affinity to the exposed phosphatidylserines lying on the outer layer of the cell membrane. Normally, phosphatidylserine lays on the inner layer

of the cell membrane. Once the cell apoptosis presents, the excessive phosphatidylserines expose to the outer layer of the cell membrane, whereby annexin V easily binds to it. PI can bind to DNA. Normally, the apoptotic cell attributes the characteristic of cell membrane integrity, cell shrinkage, and unexposed intracellular components. In contrast, the necrosis cell attributes the character of cell membrane integrity loss, cell swelling, and exposed intracellular components. According to these characteristics, PI strongly binds with high affinity to exposed DNA in necrosis, whereby it can categorize the cell death subtype, especially, apoptosis.

2. The determination of cell apoptosis by using Annexin V/PI staining

HUVECs were seeded 5×10^4 cells per well in a 24 well plate and incubated overnight. Cells were treated with the indicated protocol. After treatment for 12 hours, Cells were harvested and determined endothelial cell (ECs) apoptosis by using Muse® Annexin V & Death cell kit (Luminex Corporation, Austin, TX, USA). In brief, 100 µL of ECs suspension 5×10^5 cells/mL was added in microcentrifuge tube that contained 100 µL of Muse® Annexin V & Dead reagent and incubated for 20 minutes. Then, the microcentrifuge tube was inserted into Guava® Muse® Cell Analyzer (Luminex Corporation, Austin, TX, USA).

The stimulatory effect of PPE on HG-induced endothelial migrative impairment

The stimulatory effect of PPE on HG-induced endothelial migrative impairment was performed by using scratch-wound assay according to the previous study (Liang, *et al.*, 2007). In brief, EA.hy926 cells and HUVECs were seeded in a 6 well-plate (NEST®) and a 24 well plate at density 2×10^5 cells/well (for EA.hy926) and 9×10^4 cells/well (for HUVECs), respectively. Cells were incubated in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C until 70-90% confluence. Then, the scratch-wound was generated by vertically scratching with a p200 pipette tip on the cell monolayer. Then, ECs were washed and treated with normal glucose (NG) alone, high glucose (HG) alone, and HG in the presence of various PPE concentrations. HG in the presence of NAC 15 µg/mL was used as a positive control of HG in the presence of PPE. Then, ECs were incubated and observed wound gap closure at 0, 6, and 12 hours. The wound gap closure was

photographed by using inverted microscopy. The percentage of wound reduction was quantified by using ImageJ and calculated according to the following equation.

$$\%\, Wound\ reduction = \left[1 - \frac{Wound\ gap\ at\ specific\ timepoint}{Wound\ gap\ at\ initial\ timepoint}\right] \times 100$$

The stimulatory effect of PPE on HG-induced angiogenesis impairment

The stimulatory effect of PPE on HG-induced angiogenesis impairment was performed by using tube formation assay according to the previous study (DeCicco-Skinner, *et al.*, 2014) (Prisco, *et al.*, 2014) and Corning® protocol. In brief, the precooled 24-well plate was coated with chilled Corning® Matrigel® Matrix 10 mg/mL for 250 µL in each well and solidified at 37°C for 30 minutes in an incubator. Then, each mixture of treatment medium with HUVECs was prepared for 300 µL, whereby each mixture contains 1.2×10^5 cells of HUVECs. There are various mixtures of PPE treatment depending on investigation purpose; NG alone, HG alone, HG in the presence of PPE, and HG in the presence of NAC. After that, the gel-coated plate was overlaid with each mixture in each well and incubated at 37°C. The tube formation was observed every 2 hours. The tubule quantity was measured by photographing with inverted microscopy and calculated by using the angiogenesis plugin in ImageJ. The peak tube formation can be observed between 4 to 8 hours based on pro-angiogenic growth factor concentration. The tube degeneration was found after 12 hours of incubation.

1. Quantitation of tube network

The quantitation of tubules was performed during the tube formation period. The visualization and quantitation of tubules were performed by using inverted microscopy and ImageJ with angiogenesis analyzer plugin, respectively.

Molecular mechanism of PPE on HG-induced angiogenesis disturbance

To determine the molecular mechanism of PPE on HG-induced angiogenesis disturbance, ECs were pretreated with NG and HG for 3 days. Subsequently, ECs were treated with NG alone, HG alone, HG in the presence of PPE, and HG in the presence of NAC in individual NG- or HG-pretreated groups. After treatment, cell lysis and Western blot were also performed as follows.

1. Sample preparation

1.1. Cell lysis and protein preparation

After treatment according to indicated protocol, ECs were placed and washed with precooled 1X PBS on ice. The intracellular protein was harvested by using NP-40 lysis buffer contained with protease inhibitor cocktail (AMRESCO, OH, USA). The cell scraper was used to detach thigh adhered ECs. Then, each lysate was collected and placed on ice, and mixed every 15 minutes for 1 hour. Then, each lysate was centrifuged at 1.2×10^4 rpm for 10 minutes. The supernatant was collected and measured the protein concentration by using the Bradford assay. After that, all samples were stored at -20^{o}C for Western blot analysis.

1.2. Determination of protein concentration by using Bradford assay

The determination of protein concentration was performed by using Bradford assay before performing protein separation and Western blot analysis. In brief, protein samples and bovine serum albumin (BSA) in NP-40 lysis buffer were added to a 96 well plate for 10 µL. The serial dilution of BSA to various concentrations was considered for plotting a graph as a standard curve. Then, the Bradford reagent was added for 200 µL in each well. The sample was incubated at room temperature for 5 minutes and measured the absorbance at 595 nm by using a microplate reader. The standard curve was plotted and calculated protein concentration with a linear equation.

2. Protein separation and Western blot analysis

2.1. The principle of Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is widely used to separate the complex protein mixture by using electrical induction. Since the distinct proteins compose the different amounts of electrical charge, the distinct proteins in the sample are then separated by their net charge, whereby it has been induced by the electrical field. Namely, an excessive anion protein is rapidly translocated on gel compared to a few anion proteins or excessive cation protein. Nevertheless, the motion velocity of each protein proportionally depends on size and shape. Therefore, SDS-PAGE then consists of two pivotal components; Sodium dodecyl sulfate (SDS)/lauryl sulfate and polyacrylamide (PAM) gel (Schägger, 2006).

SDS is a reagent that breaks down the disulfide bond and unfolds the tertiary protein structure into a subunit structure. SDS-binding protein impacts protein net charge reversion. Namely, SDS binding provides a negative charge (anion), which causes the excessive positive-charged protein structure to move across the gel easily. Therefore, the motion velocity of each protein proportionally depends on size instead of shape and electrical charge of protein (Schägger*, et al.*, 1987).

PAM gel is a chemical crosslinked gel that provides protein separative ability. The motion velocity of protein proportionally depends on molecular weight (kDa) due to the chemical interaction and interferent inability of PAM gel on protein movement (Schägger *, et al.*, 1987).

2.2. Protein separation by using SDS-PAGE

Acrylamide gel was prepared by adding SDS buffer (pH 8.8) in DI water to 30% acrylamide. Polyacrylamide was subsequently polymerized by adding 10% ammonium persulfate (AP) into the mixed acrylamide solution. The N,N,N',N' – tetramethylethylenediamine (TEMED) was immediately added and dispensed in gel casting. Butanol was overlaid on the dispensed gel in casting. The separating gel was solidified at room temperature for 20 minutes. After that, the excessive solution was discarded, washed, and filled with stacking gel. The stacking gel was prepared as

same as the previously indicated procedure except for the SDS buffer, SDS buffer pH 6.8 was replaced instead of SDS buffer pH 8.8. Then, the stacking gel solution was overlaid on the separating gel. The plastic comb was immediately inserted into the stacking gel and solidified at room temperature for 20 minutes. The plastic comb was removed and washed with DI water twice. The protein sample was mixed with the Laemmli sample buffer (BioRad®) and 2-Mercaptoethanol. The mixture was loaded into each well for 20 µL. Then, the electrical induction was conducted in 1X SDS-PAGE running buffer at a certain voltage (100 V for 10 minutes and 120 V for 90 minutes, respectively). The cassette was disassembled and the gel was gently removed for Western blot analysis.

2.3. The principle of Western blot

Western blot is commonly performed for protein identification by using specific antibody interaction. The separated protein band by SDS-PAGE is transferred to the membrane before specific antibody exposure. The embedded protein band in a gel is transferred to nitrocellulose or polyvinylidene difluoride (PDVF) membrane by using electrical induction. Briefly, the gel and membrane were assembled with filter paper, pad, and supported grid as a sandwich, whereby it is immersed in the transferred buffer and transferred to the membrane by electrical induction. The transferred protein on the membrane is detected by using a protein-specific antibody. Briefly, the primary specific antibody was dispensed on the membrane and incubated at a certain duration. The secondary specific antibody with the enzyme linkage is subsequently added and visualized by appropriate methods.

2.4. Protein detection by using Western blot analysis

The embedded protein in the gel was transferred to the Immobilon-P PVDF membrane (Millipore™). This membrane was immersed in methanol for 5 minutes and incubated in a cold transfer buffer for 10 minutes before use. The gel and a PVDF membrane were assembled in cassette and placed in a transfer buffer-contained tank. The transfer protein was performed by using electrical induction at 200 mA for 90 minutes. Then, the membrane was washed with 1 X TBST buffer thrice. Non-fat dry milk (skim milk) 5% in 1 X TBST buffer was added and thoroughly agitated for

blocking non-specific binding at room temperature. After that, the membrane was washed with 1X TBST buffer thrice for 5 minutes. Then, the primary Akt, phospho-Akt, p38 MAPK, phospho-p38 MAPK, JNK, phospho-JNK, ERK, phospho-ERK, p53, Bax, caspase 9, caspase 3, and Bcl 2 specific antibody was added in individual membrane and incubated overnight. The Beta-actin specific antibody was used as internal protein control. Each primary specific antibody was diluted at the optimal concentration (1:1000) in 1% (w/v) skim milk 1 X TBST buffer before adding it to the membrane. Then, the membrane was washed with 1 X TBST buffer thrice for 5, 10, and 15 minutes, respectively. The membrane was incubated with HRP-conjugated secondary antibody in 1% (w/v) skim milk 1 X TBST buffer (1:2000) for 1 hour at room temperature. Then, the membrane was washed with 1 X TBST buffer thrice at 5, 10, and 15 minutes, respectively. Lumina™ Crescendo Western HRP was used as an HPR substrate for detecting interested-protein bands. The image was developed by placing the membrane in Chimidoc™ XRS (Bio-rad, CA, USA). The molecular weight of protein band intensity was confirmed to molecular weight indicating in marker lane.

The stimulatory effect of PPE on HG-reduced pro-angiogenic growth factors secretion

Since pro-angiogenic growth factors stimulate angiogenesis by activating tube-like endothelial formation, the pro-angiogenic growth factor secretion is then emphasized. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and epidermal growth factors (EGF) are essential pro-angiogenic growth factors that stimulate vessel formation (Mizia-Malarz*, et al.,* 2008). The stimulatory effect of PPE on HG-reduced VEGF, bFGF, and EGF secretion was determined by using sandwich enzyme-linked immunosorbent assay (ELISA) as follows.

1. The principle of ELISA

ELISA is a common technique for detecting the protein-ligand in a liquid sample by using antigen-antibody interaction. There are many subtypes of ELISA techniques such as direct, sandwich, competitive, and reverse ELISA. However, there

is no difference in terms of basic principles. Briefly, the sample is coated by the interaction of the pre-coated antibody in a 96 well plate. the protein-specific antibody is subsequently added to the sample-coated 96 well plate. The amount of protein in the sample can be detected by the protein-specific antibody. Namely, the high amount of protein in the sample can intensively recruit specific antibody binding more than the low amount of protein. Then, the presence of the protein is detected by adding an enzyme-conjugated secondary antibody and substrate (Lequin, 2005).

2. Determination of pro-angiogenic growth factors by using sandwich ELISA technique

Sandwich ELISA was performed according to the previous protocol (Granato, *et al.*, 2004). In brief, VEGF, bFGF, and EGF were determined by using human VEGF ELISA development kit, human FGF-basic standard ABTS ELISA development kit, and human EGF standard ABTS ELISA development kit (PeproTech Inc., Rocky Hill, NJ, USA), respectively. After treatment according to indicated protocol, the supernatant was collected and discarded cell debris by centrifugation. Then, the supernatant was incubated in pre-blocked capture antibody-coated 96-well plate for 2 hours at room temperature. Then, the detection antibody was added and incubated for 2 hours at room temperature. After that, the avidin-HRP-conjugated anti IgG was added and incubated for 30 minutes at room temperature. After adding a substrate, the luminescence was determined by using a microplate reader at 450/605 nm.

Statistical analysis

All data is shown as mean ± SEM and analyzed the significant difference by using ANOVA with appropriate post-hoc comparison analysis. A p-value < 0.05 was considered as a statistically significant difference. The statistical analysis was performed by using commercially available software (GraphPad Prism version 7).

CHAPTER IV
RESULTS

The modeling of high glucose condition

The endothelial cell (ECs) viability and intracellular ROS generation were determined to evaluate the minimum glucose concentration as HG condition in further experiments. EA.hy926 cells were treated with glucose 5.5, 15, 25, and 35 mM (110, 300, 500, and 700 mg/dL) in 0.5% FBS complete medium. Glucose 5.5 mM represents normal glucose (NG) as a control. The MTT viability assay was performed according to indicated protocol. The results demonstrated glucose 35 mM significantly reduced ECs viability compared to NG both 24 and 48 hours. Interestingly, HG (glucose 15, 25, and 35 mM) significantly reduced ECs proliferation compared to NG at 48 hours (Figure 8A). All data is shown in mean ± SEM, * $p < 0.05$ (One way-ANOVA, 3-time independent, n=9).

EA.hy926 cells were also treated with glucose 5.5, 25, 35, and 45 mM in 0.5% FBS complete medium for determining intracellular ROS overproduction. Glucose 5.5 mM represents normal glucose (NG) as a control. NG in the presence of hydrogen peroxide (H_2O_2) 500 µM represents positive control of the experimental setting. The results demonstrated glucose 45 mM significantly enhanced intracellular ROS generation compared to NG both 24 and 48 hours. Interestingly, Glucose 35 mM also significantly enhanced intracellular ROS compared to NG at 48 hours. NG in the presence of H_2O_2 500 µM also expressed the enhancement of intracellular ROS generation compared to NG both 24 and 48 hours with significant difference (Figure 8B). All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 3-time independent, n=9).

According to the results, glucose 35 mM represents the minimum concentration that reduced ECs viability and increased intracellular ROS compared to NG with a significant difference. Glucose 35 mM was used as an HG model in a further experiment, whereby it potentially provided the reversible effect of PPE treatment. Additionally, the declination of ECs viability by glucose 35 mM was also confirmed that was not a consequence of hyperosmolarity by using mannitol 35 mM as the osmolarity control, because mannitol has a similar chemical formula to glucose.

The results illustrated there is no significant difference between mannitol 35 mM and vehicle. Interestingly, glucose 35 mM significantly reduced ECs viability compared to vehicle and mannitol 35 mM. There is also no significant difference between glucose 5.5 mM (NG) and mannitol 35 mM (Figure 8C). It suggests the impairment of ECs viability by glucose 35 mM compared to NG was not a consequence of the hyperosmolar effect. All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, n=3).

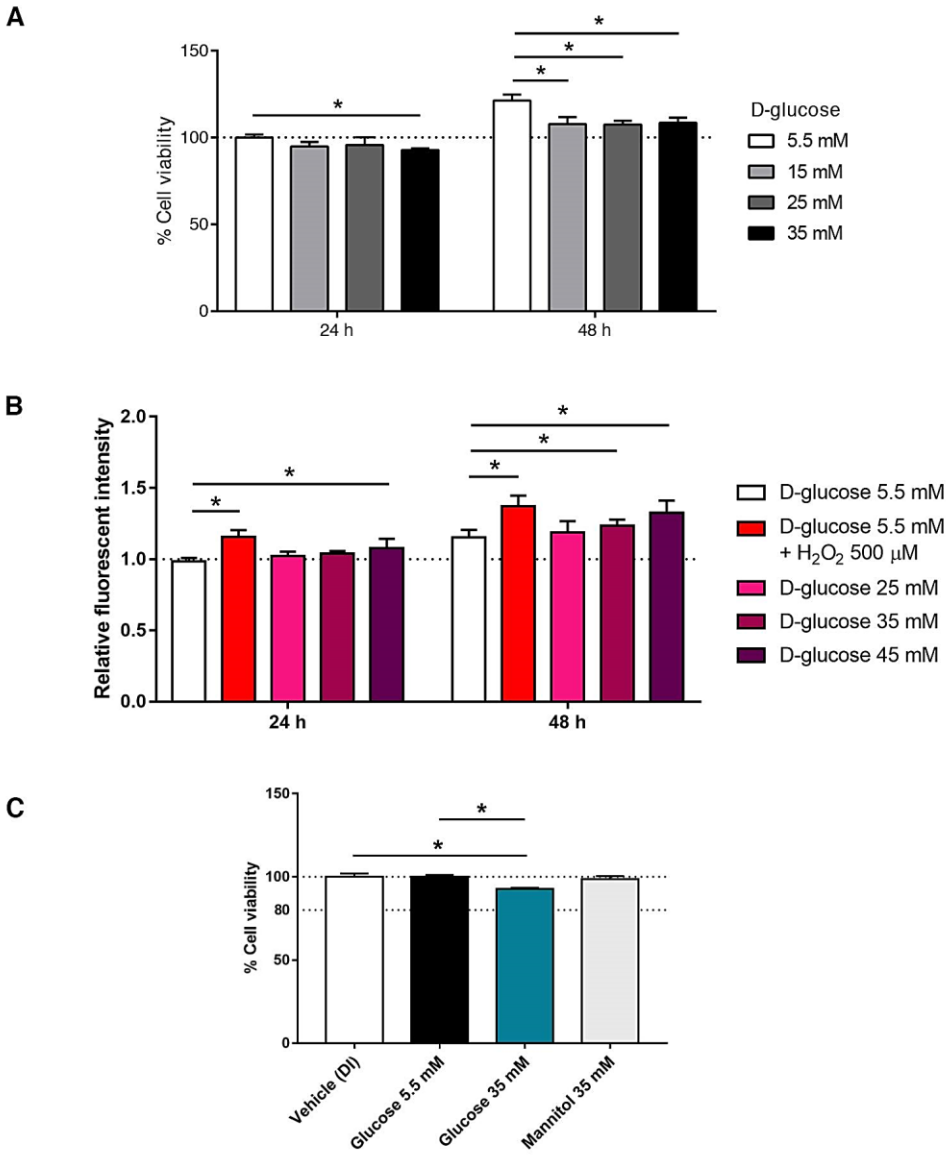

Figure 8 The model of high glucose condition.

(A) EA.hy926 cells were treated with the indicated condition and determined the endothelial cell viability by using MTT viability assay. **(B)** EA.hy926 cells were also treated with the indicated condition and determined intracellular ROS by using a DCFDA reagent. **(C)** EA.hy926 cells were treated with the indicated condition and determined ECs viability at 24 hours. Each bar represents in mean ± SEM * $p < 0.05$ **(A, B)** (One-way ANOVA, 3-time independent, n=9), **(C)**(One-way ANOVA, n=3).

The optimization of PPE concentrations

The ECs viability was determined to evaluate the optimal PPE concentrations in NG. EA.hy926 were treated with PPE 0, 5, 10, 20, and 40 µg/mL in 0.5% FBS complete medium. PPE 0 µg/mL represents control. The MTT viability assay was performed according to indicated protocol. The results demonstrated PPE 5 and 10 µg/mL significantly enhance ECs viability compared to control at 48 hours. PPE (5, 10, 20, and 40 µg/mL) did not express the enhancement of ECs viability compared to control at 24 hours. However, PPE did not also reduce ECs viability by less than 80% compared to control at 24 hours. According to the results, PPE 5 and 10 µg/mL were used for investigating in further experiments as optimal PPE concentrations. These optimal concentrations significantly increased ECs viability and did not show ECs cytotoxicity, where it did not decrease ECs viability less than 80% (Figure 9). All data is shown in mean ± SEM, * $p < 0.05$ (One way-ANOVA, 3-time independent, n=9)

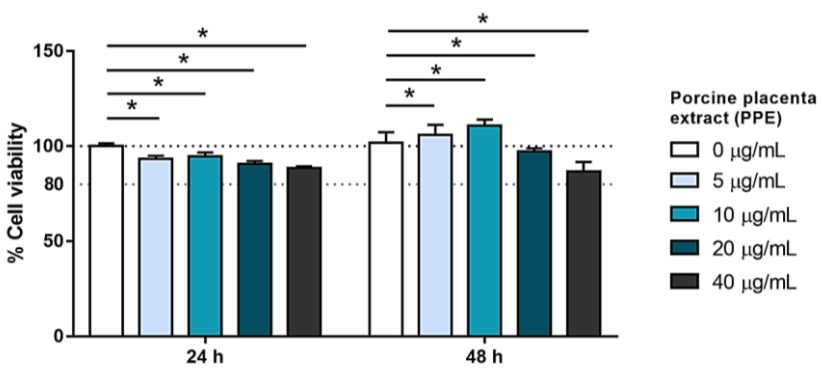

Figure 9 The optimization of PPE concentrations

EA.hy926 cells were treated with the indicated condition and determined the endothelial cell viability by using the MTT viability assay. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 3-time independent, n=9).

PPE attenuated HG-induced intracellular ROS overproduction

The inhibitory effect of PPE on HG-induced intracellular ROS overproduction was determined by using a DCFDA reagent. HUVECs and EA.hy926 cells were treated with NG alone, NG in the presence of H_2O_2 (500 µM for EA,hy926 cells and 100 µM for HUVECs), HG alone, HG in the presence of PPE (2.5, 5, 10, 15 µg/mL), and NG in the presence of N-acetylcysteine (NAC) 15 µg/mL for 12 hours. NG in the presence of H_2O_2 represents positive control of the experimental setting. HG in the presence of NAC represents positive control of PPE treatment. The intracellular ROS determination by using DCFDA reagent was performed according to indicated protocol. The results demonstrated HG alone significantly enhanced intracellular ROS compared to NG alone, whereby NG in the presence of H_2O_2 also elucidated the enhancement of intracellular ROS compared to NG alone. Remarkably, HG in the presence of PPE 15 µg/mL significantly reduced intracellular ROS compared to HG alone, whereby HG in the presence of NAC 15 µg/mL also showed the reduction of intracellular ROS compared to HG alone with significant difference. It suggests PPE attenuated HG-induced intracellular ROS generation (Figure 10A and 10B). All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

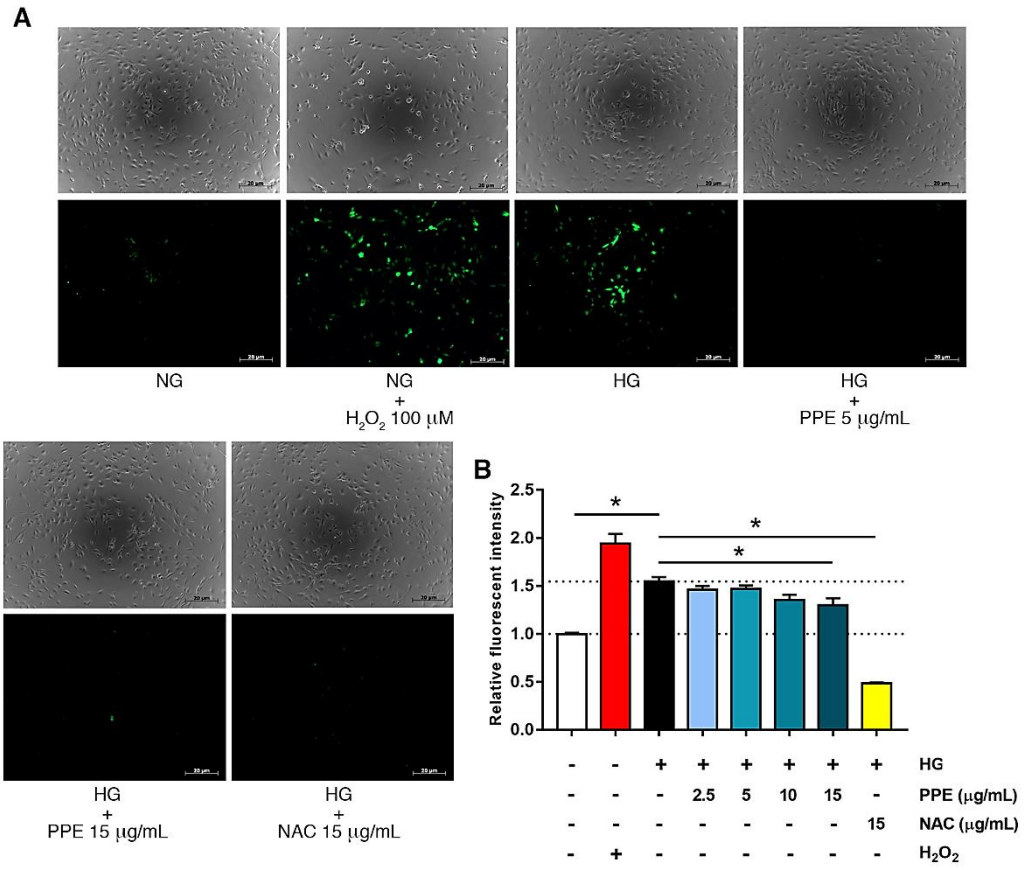

Figure 10 PPE attenuated HG-induced intracellular ROS overproduction.

HUVECs were treated with the indicated condition and determined intracellular ROS by using DCFDA reagent at 12 hours. The intracellular ROS was observed and photographed by using inverted microscopy with a FITC filter. **(B)** EA.hy926 cells were also treated with the indicated condition and determine intracellular ROS by using DCFDA reagent at 12 hours. The intracellular ROS was detected by using a microplate reader at 485 nm excitation/535 nm emission. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6).

PPE improved HG-induced endothelial viable impairment

The stimulatory effect of PPE on HG-induced endothelial viable impairment was determined by using the MTT viability assay. HUVECs and EA.hy926 cells were treated with NG alone, HG alone, and HG in the presence of PPE (2.5, 5, 10, 15 µg/mL) for 24 hours. The ECs viable determination by using MTT viability assay was performed according to indicated protocol. The results elucidated HG alone significantly reduced ECs viability compared to NG alone both in HUVECs (Figure 11A) and EA.hy926 cells (Figure 11B), whereby HG in the presence of PPE 5, 10, and 15 µg/mL significantly enhance ECs viability compared to HG alone. It suggests PPE improved HG-induced endothelial viable impairment. All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

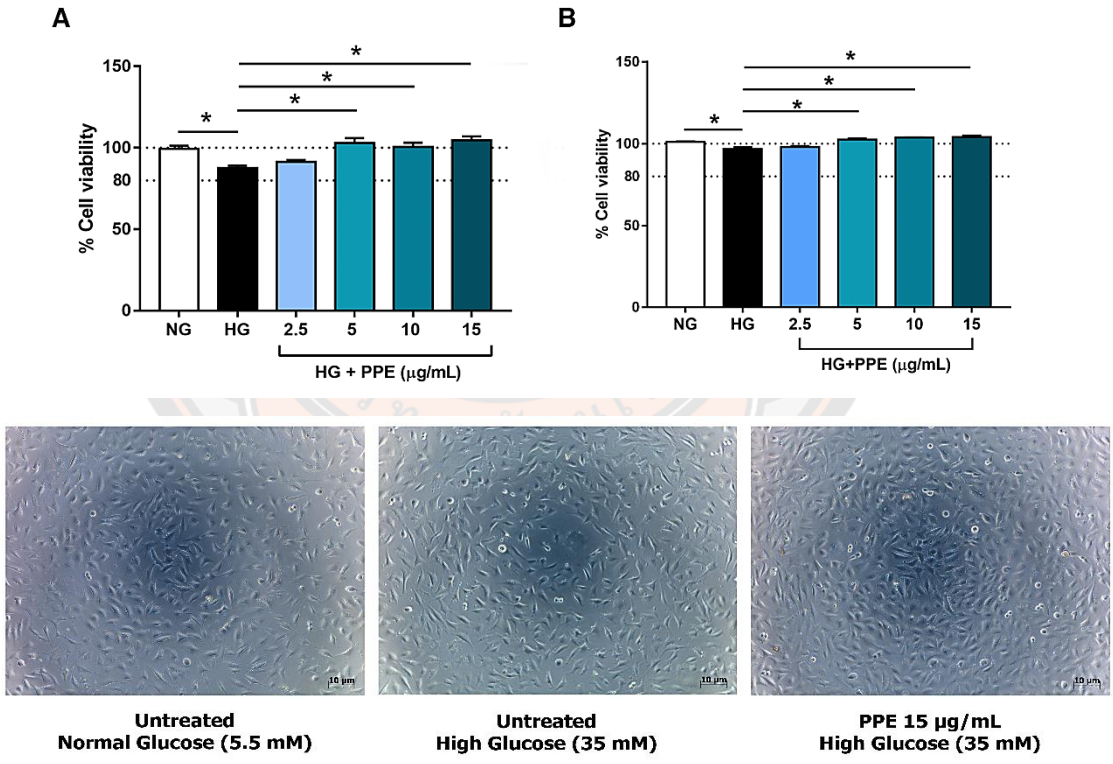

Figure 11 PPE improved HG-induced ECs viable impairment.

(A) HUVECs and **(B)** EA.hy926 cells were treated with the indicated condition and determined endothelial cell viability by using the MTT viability assay at 24 hours. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6).

PPE improved HG-induced endothelial viable impairment through the inhibition of ROS-mediated endothelial apoptosis

To confirm the stimulatory effect of PPE on HG-induced ECs viable impairment. The ECs apoptosis was determined by using annexin V/PI staining. HUVECs were treated with NG alone, HG alone, HG in the presence of PPE (2.5, 5, 10, 15 µg/mL), and HG in the presence of NAC 15 µg/mL for 24 hours. HG in the presence of NAC 15 µg/mL represents positive control of PPE treatment. The annexin V/PI staining was performed according to indicated protocol. The results elucidated HG alone significantly enhanced the percentage of total apoptotic cells compared to NG alone, whereby HG alone also significantly reduced the percentage of live cells compared to NG alone. HG in the presence of PPE 5, 10, and 15 µg/mL significantly reduced the percentage of total apoptotic cells compared to HG alone, whereby HG in the presence of PPE 5, 10, and 15 µg/mL significantly enhanced the percentage of live cells compared to HG alone. HG in the presence of NAC 15 µg/mL also elucidated the reductive percentage of total apoptotic cells and augmentative percentage of live cells with significant differences (Figure 12A and 12B). It suggests PPE improved HG-induced ECs viable impairment through the inhibition of ROS-mediated ECs apoptosis. All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 3-time independent, n=6)

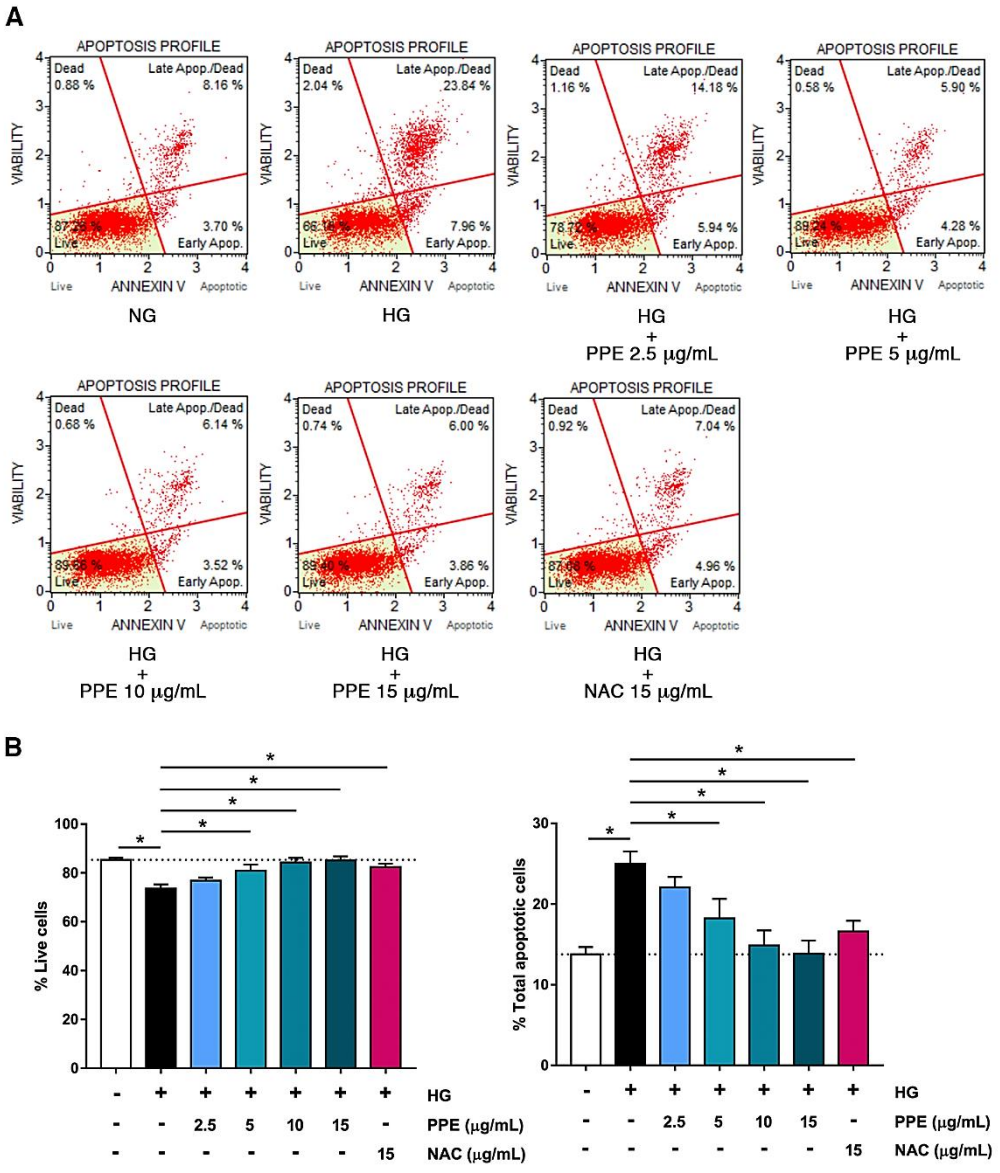

Figure 12 PPE improved HG-induced endothelial viable impairment through the inhibition of ROS-mediated endothelial apoptosis.

HUVECs were treated with the indicated condition and determined endothelial cell apoptosis by using annexin V/PI staining. **(A)** The dot plots represent apoptotic profiles and **(B)** The bar graphs represent the percentage of live cells and total apoptotic cells. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 3-time independent, n=6)

PPE improved HG-induced endothelial migrative impairment

The stimulatory effect of PPE on HG-induced ECs migrative impairment was determined by using the scratch-wound assay. HUVECs and EA.hy926 cells were treated with NG alone, HG alone, HG in the presence of PPE 5 and 15 µg/mL, and HG in the presence of NAC 15 µg/mL. HG in the presence of NAC 15 µg/mL represents positive control of PPE treatment. The percentage of wound reduction was determined according to indicated protocol. The results indicated HG alone significantly reduced the percentage of wound reduction compared to NG alone both in EA.hy926 cells (Figure 13) and HUVECs (Figure 14). Interestingly, HG in the presence of PPE 15 µg/mL significantly enhanced the percentage of wound reduction compared to HG alone both in HUVECs and EA.hy926 cells. HG in the presence of NAC 15 µg/mL also elucidated the augmentative percentage of wound reduction in HUVECs. It suggests PPE improved HG-induced ECs migrative impairment. All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

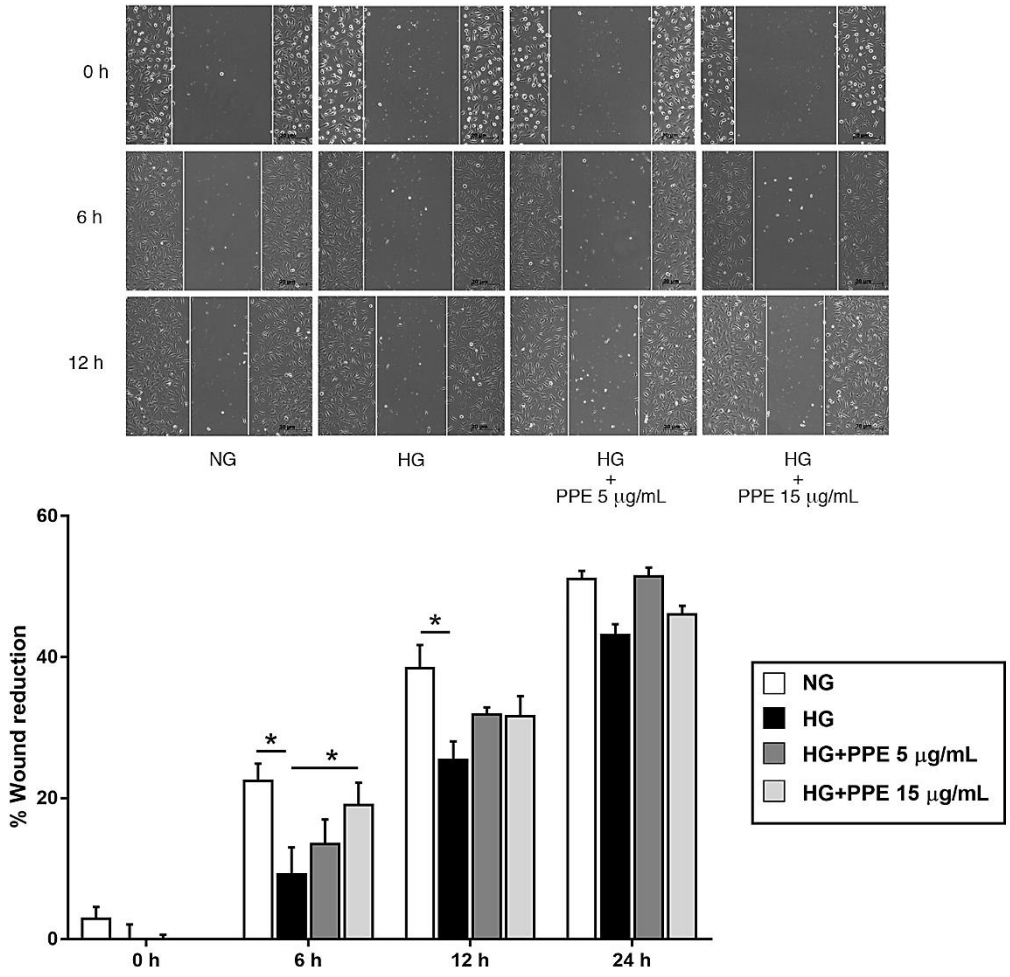

Figure 13 PPE improved HG-induced EA.hy926 cells migrative impairment.

EA.hy926 cells were treated with the indicated condition and determined the percentage of wound reduction. The scratch wound was generated before treatment. The percentage of wound reduction was calculated according to indicated equation. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 3-time independent, n=9)

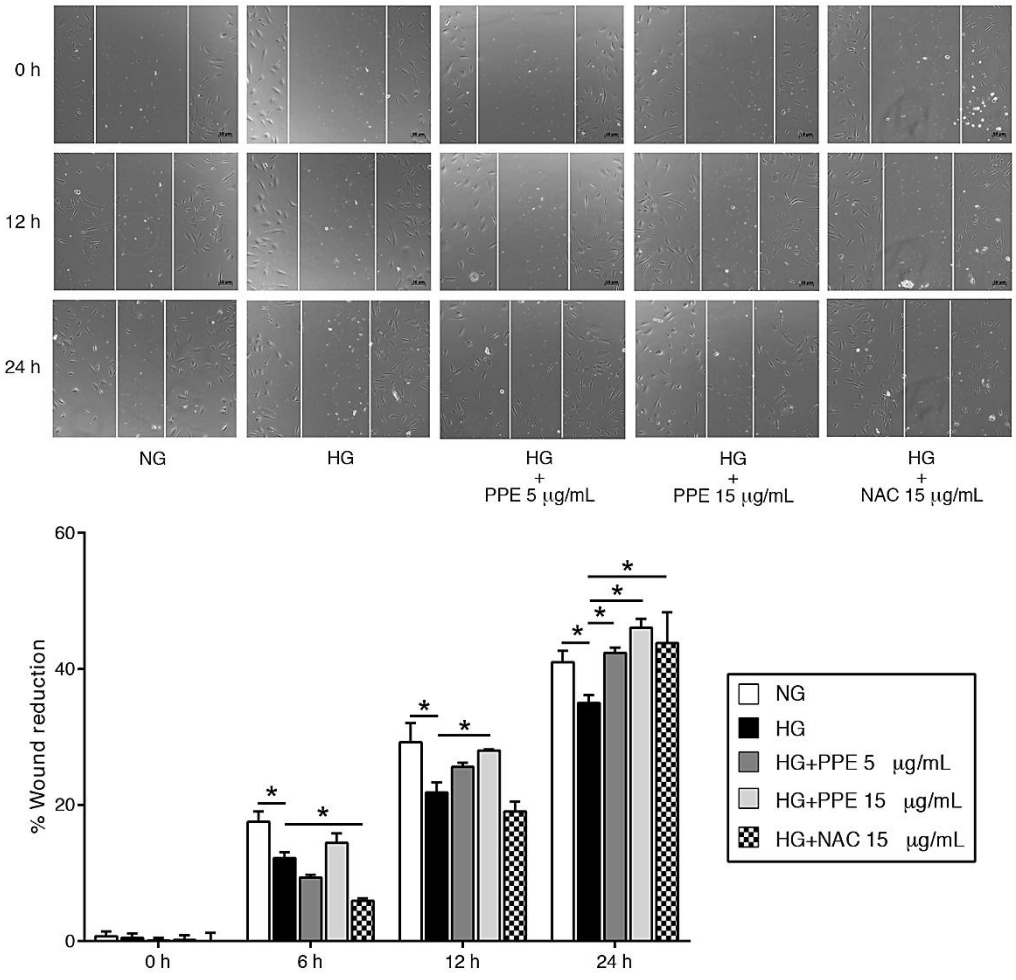

Figure 14 PPE improved HG-induced HUVECs migrative impairment.

HUVECs were treated with the indicated condition and determined the percentage of wound reduction. The scratch wound was generated before treatment. The percentage of wound reduction was calculated according to indicated equation. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

PPE improved HG-induced angiogenesis impairment

The stimulatory effect of PPE on HG-induced angiogenesis impairment was determined by using a tube formation assay. The experiment was performed according to indicated protocol. HUVECs were treated with NG alone, HG alone, HG in the presence of PPE 5 and 15 µg/mL, and HG in the presence of NAC 15 µg/mL. HG in the presence of NAC 15 µg/mL represents positive control of PPE treatment. The number of tubules was determined according to indicated protocol. The results illustrated HG alone significantly decreased the number of tubules compared to NG alone, whereas HG in the presence of PPE 15 µg/mL significantly increased the number of tubules compared to HG alone. HG in the presence of NAC µg/mL also presented the augmentative number of tubules compared to HG alone. It suggests PPE improved HG-induced angiogenesis impairment (Figure 15). All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

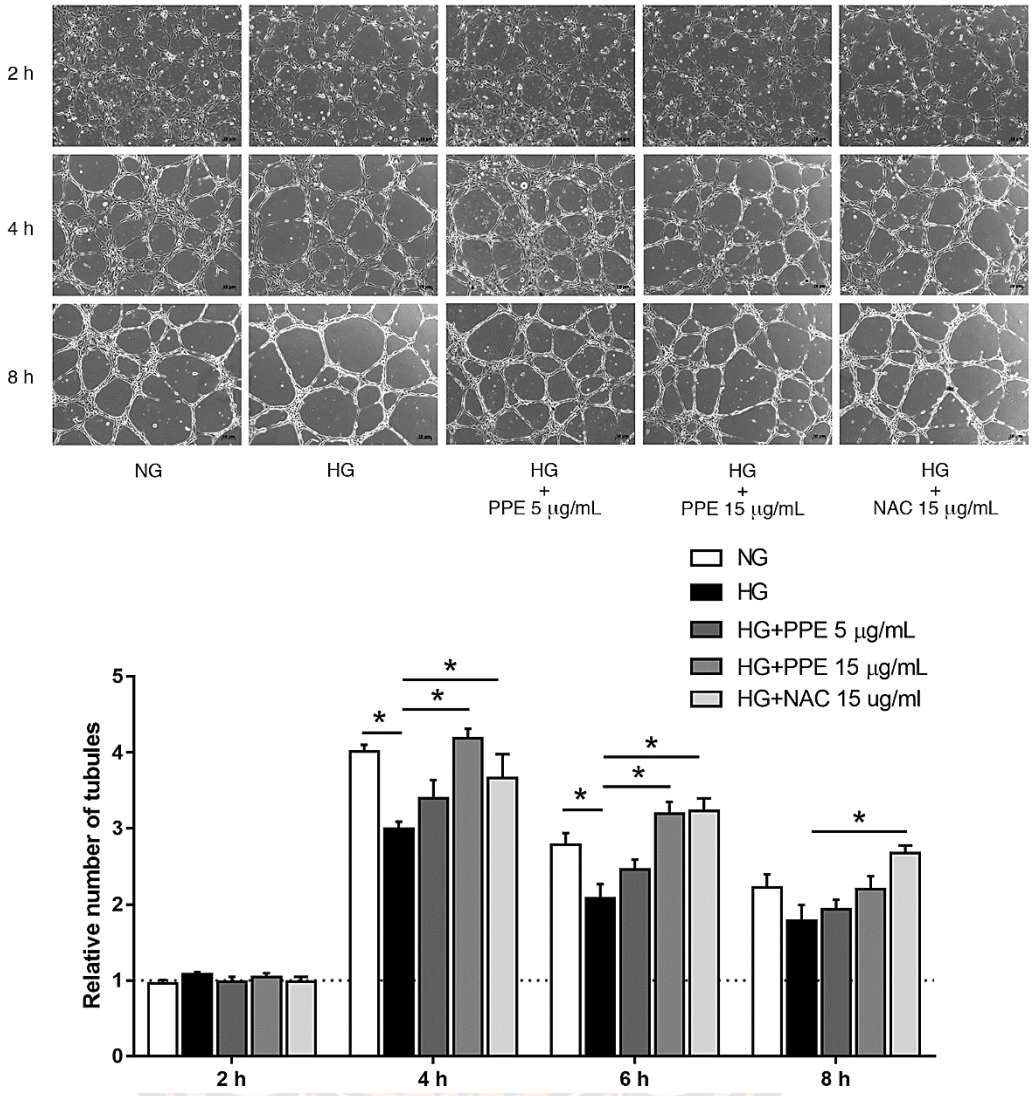

Figure 15 PPE improved HG-induced angiogenesis impairment.

HUVECs were treated with the indicated condition and determined the number of tubules every 2 hours. The tube formation assay was conducted according to indicated protocol. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

PPE ameliorated HG-reduced pro-angiogenic growth factor secretion

The stimulatory effect of PPE on HG-reduced pro-angiogenic growth factors secretion was determined by using the sandwich ELISA technique. HUVECs were treated with NG alone, HG alone, HG in the presence of PPE (3.75, 7.5, and 15 µg/mL), and HG in the presence of NAC 15 µg/mL for 48 hours. HG in the presence of NAC 15 µg/mL represents positive control of PPE treatment. VEGF, bFGF, and EGF in the supernatant were determined according to the indicated protocol. The results illustrated HG alone potentially decreased bFGF level in supernatant compared to NG alone, whereas HG in the presence of PPE potentially increased bFGF level in supernatant compared to HG alone. However, there is no difference in EGF level in the supernatant of each group (Figure 16). Unfortunately, the VEGF level could not be detected in the supernatant. It suggests PPE potentially ameliorated HG-induced pro-angiogenic growth factors secretion especially bFGF. All data is shown in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

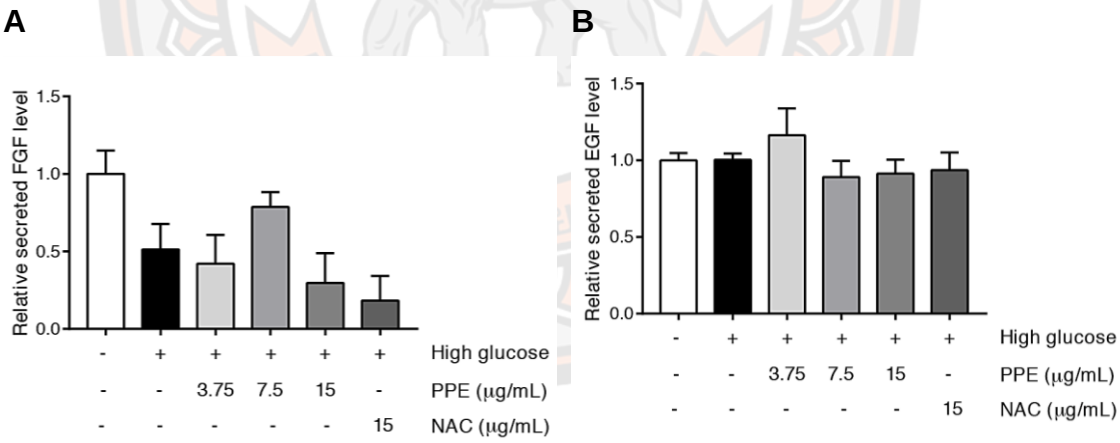

Figure 16 PPE ameliorated HG-reduced pro-angiogenic growth factor secretion. HUVECs were treated with the indicated condition for 2 days. Then, the supernatant was collected and determined (A) bFGF, (A) EGF levels by using the sandwich ELISA technique. Each bar represents in mean ± SEM * $p < 0.05$ (One way-ANOVA, 2-time independent, n=6)

PPE recovered HG-reduced PI3K/Akt/ERK1/2 activation

To investigate the molecular mechanisms of PPE on HG-induced angiogenesis impairment, the pro-survival signaling pathway was emphasized. ECs were treated with NG alone, HG alone, HG in the presence of PPE (3.75, 7.5, and 15 µg/mL), and HG in the presence of NAC µg/mL as a positive control of PPE treatment. The phospho-PI3K, phospho-Akt, Akt, phospho-ERK1/2, ERK1/2, and actin were determined by using Western blot analysis. The results illustrated HG alone potentially reduced phosphorylation of PI3K, Akt, and ERK1/2 activation compared to NG alone, whereas HG in the presence of PPE potentially enhanced phosphorylation of PI3K, Akt, and ERK1/2 activation compared to HG alone. HG in the presence of NAC also illustrated the potential of augmentative activation of PI3K, Akt, and ERK1/2. It suggests PPE potentially recovered HG-reduced PI3K/Akt/ERK1/2 activation. The ratio of phosphor-protein band intensity and total-protein band intensity was represented in the individual lane (Figure 17).

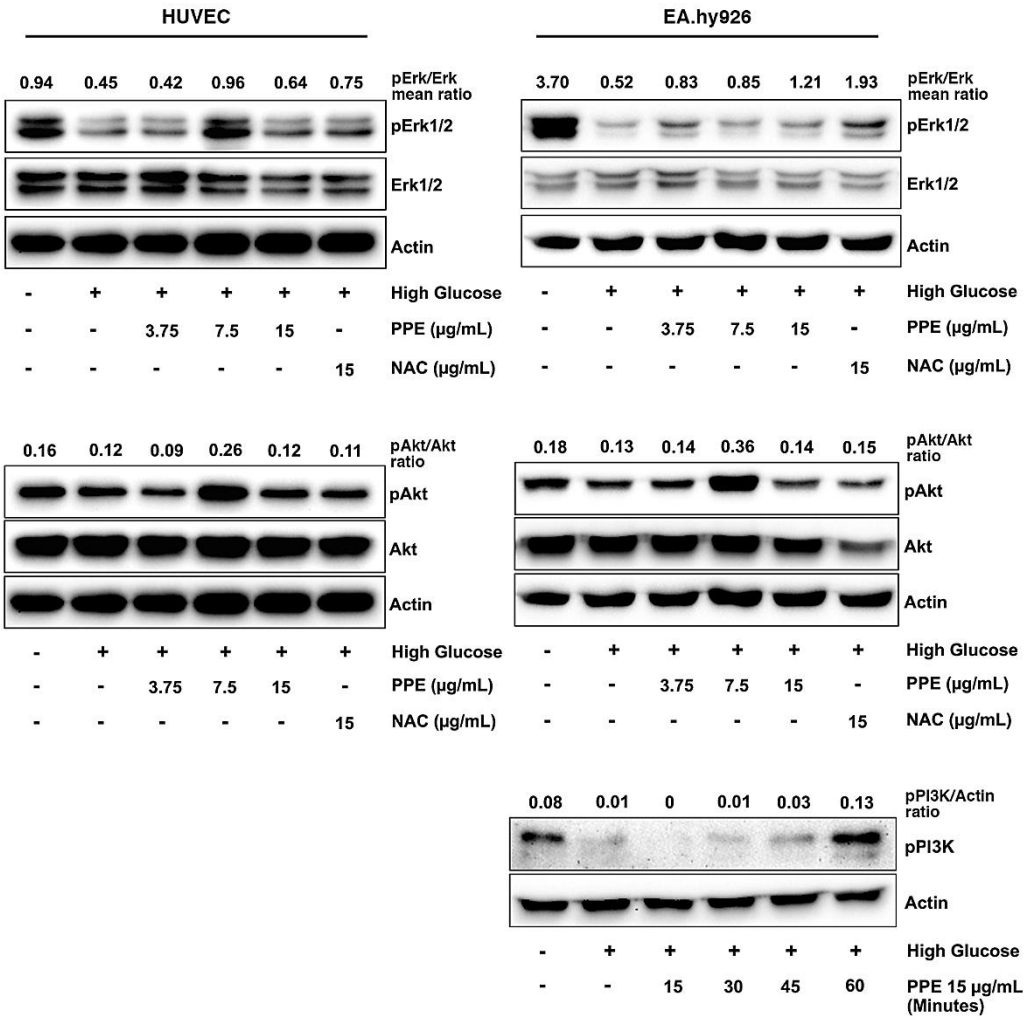

Figure 17 PPE recovered HG-reduced PI3K/Akt/ERK1/2 activation.

ECs were treated with the indicated condition. The ECs were lysed and the pro-survival signaling pathway was determined by using Western blot analysis. The ratio of phosphor-protein band intensity and total-protein band intensity was represented in the individual lane. The ratio of phospho-PI3K and actin was also represented in the individual lane.

PPE attenuated HG-increased apoptotic regulatory molecules

The inhibitory effect of PPE on HG-increased apoptotic regulatory molecules was determined by using Western blot analysis. ECs were treated with NG alone, HG alone, HG in the presence of PPE (3.75, 7.5, and 15 µg/mL), and HG in the presence of NAC µg/mL as a positive control of PPE treatment. The results illustrated HG alone potentially increased p53, Bax, cleaved caspase 9, and cleaved caspase 3 levels compare to NG alone, whereas it potentially decreased Bcl 2 level compared to NG alone. Remarkably, HG in the presence of PPE potentially reduced p53, Bax, cleaved caspase 9, and cleaved caspase 3 levels compared to HG alone, whereby it potentially increased Bcl 2 level compared to HG alone. It suggests PPE attenuated HG-increased apoptotic regulatory molecules. The ratio of apoptotic regulatory protein band intensity and actin band intensity was represented in the individual lane. The Bax/Bcl 2 ratio of NG alone, HG alone, HG in the presence of PPE and NAC are 6.90, 7.16, 6,66, 5.23, 2.88, and 3.17, respectively (Figure 18)

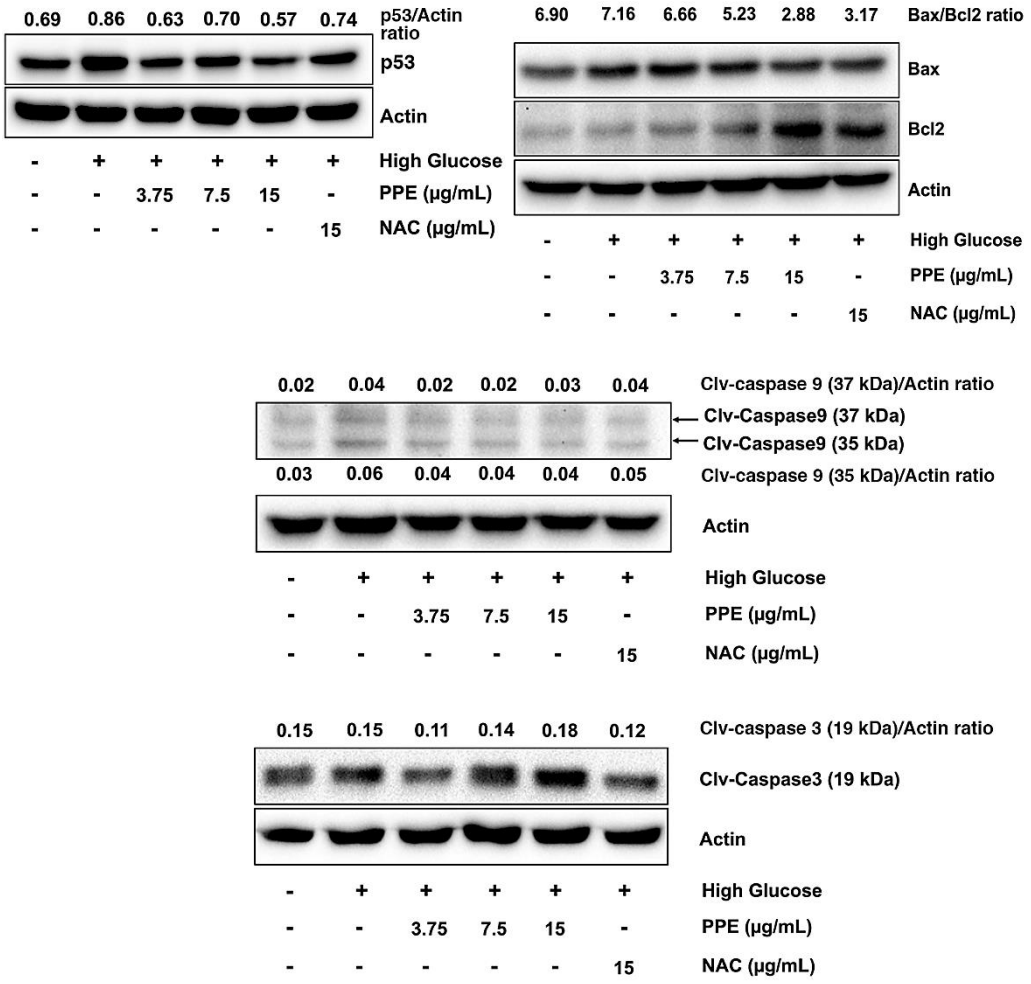

Figure 18 PPE attenuated HG-increased apoptotic regulatory molecules.

ECs were treated with the indicated condition. The ECs were lysed, and the apoptotic regulatory molecules were determined by using Western blot analysis. The ratio of apoptotic regulatory molecules and actin was represented in the individual lane.

PPE involved the stress-sensitive signaling pathway

The stress-sensitive signaling pathway was determined by using Western blot analysis. ECs were treated with NG alone, HG alone, HG in the presence of PPE (3.75, 7.5, and 15 µg/mL), and HG in the presence of NAC µg/mL as a positive control of PPE treatment. The phospho-p38 MAPK, p38 MAPK, phospho-JNK, and JNK were determined according to the indicated protocol. The results illustrated HG alone potentially increased phosphorylation of p38 MAPK and JNK activation. However, PPE also showed the activation of p38 MAPK and JNK, whereby it gradually decreased over time. It suggests PPE involved the stress-sensitive signaling pathway (Figure 19).

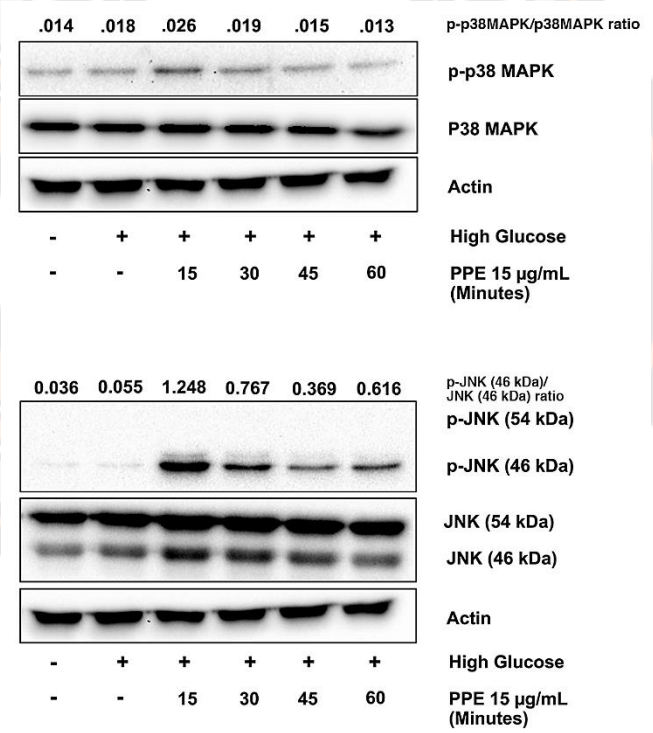

Figure 19 PPE involved the stress-sensitive signaling pathway.

EA.hy926 cells were treated with the indicated condition. Cells were lysed, and the stress-sensitive signaling pathway was determined by using Western blot analysis. The ratio of phosphor-protein band intensity and total-protein band intensity was represented in the individual lane.

CHAPTER V
DISCUSSIONS AND CONCLUSIONS

The angiogenesis disturbance has been implied to be the microvascular complication of DM, whereby it has been classified in two statements; excessive angiogenesis and angiogenesis impairment (Cheng, *et al.*, 2015). Excessive angiogenesis accounts for a pathophysiological process of proliferative diabetic retinopathy in the diabetic patient, whereas the angiogenesis impairment accounts for a pathophysiological process of delayed wound healing in diabetic patients (Fadini, *et al.*, 2019). Even there is a different response of ECs to HG exposure, this mechanistic insight of the angiogenesis paradox is still unclear. Some studies have indicated a different pathophysiologic response of distinct local ECs to HG (Kota, *et al.*, 2012). However, the angiogenesis impairment is emphasized in this study, because angiogenesis impairment is a pivotal factor that leads to delayed wound healing in poorly controlled diabetic patients (Polverini, 2011). HG-induced intracellular ROS overproduction has been remarked as a key factor of HG-induced ECs dysfunction (Shenouda, *et al.*, 2011; Szewczyk, *et al.*, 2015). HG-induced ECs dysfunction in terms of viable, migrative, and angiogenesis impairment has also been implicated as a consequence of intracellular ROS overproduction (Rezabakhsh, Ahmadi, *et al.*, 2017; Zhu, *et al.*, 2015a). Interestingly, PPE has been demonstrated as a wound healing accelerator and ROS inhibitor in previous studies (Heo , *et al.*, 2018; C.-H. Wu, *et al.*, 2003). Therefore, PPE may provide the improving potential of angiogenesis impairment through intracellular ROS inhibition and ECs viable and migrative stimulation in HG. In this study, ECs were treated with PPE in HG to investigate the stimulatory effect of PPE on angiogenesis. HG alone clarified the evidence of ECs viable, migrative, and angiogenesis impairment through the intracellular ROS increment. The major finding in this study is the inhibitory effect of PPE on HG-induced ROS overproduction, whereby it preserved ECs viability, migration, and angiogenesis by showing the recovery of PI3K/Akt/ERK1/2 activation. Additionally, the inhibitory effect of PPE on HG-induced ROS overproduction also attenuated ECs apoptosis by showing the regulation of apoptotic regulatory molecules (Figure 20).

The intracellular ROS overproduction plays a role in HG-induced glucotoxicity. The excessive glucose influx increases intracellular ROS by depriving redox pathway-related molecules (Z. Zhang, *et al.*, 2012). Moreover, the NADPH oxidase (NOX)-derived ROS in ECs has been implicated a major consequence of HG exposure. This excessive ROS directly disrupts DNA and induces GAPDH blockage (X. Du , *et al.*, 2003). Since ROS-induced GAPDH blockage inhibits glyceraldehyde 3-phosphate (G3P) conversion, the upstream glycolysis converts to alternative pathways that are defined as glucotoxicity; polyol pathway, glucosamine pathway, protein kinase C (PKC) pathway, and advanced-glycation end product (AGE) pathway (Ighodaro, 2018; Incalza, *et al.*, 2018; Pricci, *et al.*, 2003). Remarkably, the homeostasis of intracellular ROS is an important key for improving HG-induced ECs dysfunction. The findings from the current study elucidate that the inhibition of HG-induced ROS overproduction preserves ECs viability, migration, angiogenesis similar to other studies (Duan, *et al.*, 2019; Li, *et al.*, 2017; Zhou, *et al.*, 2015).

Angiogenesis comprises many processes to form a new capillary. ECs are initially activated by pro-angiogenic signals, whereby it induces ECs proliferation and migration for a new tube formation. Angiogenesis impairment has been clarified as a consequence of HG-deprived ECs viability and migration, which is a consequence of HG-induced intracellular ROS overproduction (Rezabakhsh , *et al.*, 2017; Rezabakhsh, Montazersaheb, *et al.*, 2017). The findings from the current study showed angiogenic growth factors and hormones improve HG-induced ECs impairment similar to other studies (Han, *et al.*, 2005; Song, *et al.*, 2014; Yang, *et al.*, 2008). PPE in this study also characterized the protein components by LC-MS/MS and searched data against the domestic porcine *Sus scofa* proteome database. The PPE contains 391 protein sequences from 447 total proteins. The analysis of DAVID bioinformatics resources elucidated that our PPE mostly comprised signal-involved proteins, phosphoproteins, and disulfide-bond proteins (Padhomchai Pumbthongthae, 2020). Accordingly, PPE in this study may consist of essential growth factors and antioxidative agents or reductant enzymes that provide bioactive functions.

The inhibition of the pro-survival signaling pathway has been illustrated as the molecular signaling of HG-induced ROS-mediated angiogenesis impairment in many studies (Duan , *et al.*, 2019; Maamoun, *et al.*, 2019; Xing, *et al.*, 2017). The

excessive ROS inhibits the PI3K/Akt signaling pathway, whereby it leads to insulin resistance and ECs survival reduction (De Nigris, *et al.*, 2015). The ROS-induced Akt downregulation also implicated ECs migrative impairment (Lamalice, *et al.*, 2007). In this study, HG reduced phosphorylation of PI3K, Akt, and ERK1/2 compared to NG, whereas PPE increased phosphorylation of PI3K, Akt, and ERK1/2 in HG. It suggests PPE ameliorated HG-reduced PI3K, Akt, and ERK1/2 activation through intracellular ROS inhibition. This improvement of the PI3K/Akt/ERK1/2 activation eventually preserved ECs viability and migration in HG. Additionally, growth factors and hormones have been elucidated as the major activator of the pro-survival signaling pathway (Kazi, *et al.*, 2009). Therefore, there is two possible pathway of PPE on HG-induced angiogenesis impairment. Firstly, PPE inhibited HG-induced ROS overproduction, whereby it eventually improved ECs viable, migrative, and angiogenesis impairment by recovering the PI3K/Akt/ERK1/2 activation. Secondly, PPE directly enhanced PI3K/Akt/ERK1/2 activation, whereby it concomitantly improved ECs viable, migrative, and angiogenesis impairment (Figure 20).

Moreover, many studies have indicated HG-induced ROS-mediated ECs apoptosis (Hou, *et al.*, 2015; N. Wu, *et al.*, 2016). The investigation of PPE on HG-induced ECs apoptosis and the apoptotic regulatory molecules such as p53, Bax, Bcl 2, cleaved caspase 9, and cleaved caspase 3 were determined to elucidate the inhibitory effect of PPE on HG-induced ROS-mediated ECs apoptosis. In this study, PPE attenuated HG-induced ECs apoptosis by showing the declination of p53, Bax, cleaved caspase 9, cleaved caspase 3 levels, and the increment of Bcl 2 level, respectively. It suggests PPE also improved ECs viable impairment through the attenuation of HG-induced ROS-mediated ECs apoptosis (Figure 20). The stress-sensitive signaling pathway was also investigated in this study. The p38 MAPK and JNK were determined by using Western blot analysis. The result indicated that PPE activated p38 MAPK and JNK pathway (Figure 19). Some studies have reported bFGF induced p38 MAPK and JNK activation, whereby it induced Akt and MMP-2 activation resulting in enhanced migration and angiogenic activity (Kim, *et al.*, 2014). VEGF has implicated the p38 MAPK activation through Scr and RAFTK/Pyk2 induction, whereby it affects ECs migration (McMullen, *et al.*, 2004). Additionally, p38 MAPK activation has been implicated ECs migration in other studies (Rousseau,

et al., 1997). However, the inhibitory effect of PPE on HG-induced ROS-mediated angiogenesis impairment is still emphasized, in which the reduction of HG-induced intracellular ROS by PPE treatment should decrease p38 MAPK and JNK activation. The activation of p38 MAPK and JNK by PPE treatment in NG should further confirm by comparing with and without p38 MAPK and JNK inhibitors.

The angiogenic growth factor secretion was also determined in this study. PPE potentially ameliorated HG-reduced bFGF and EGF secretion, which are pivotal signals for angiogenesis activation. The VEGF has been clarified the stimulatory effect of pro-angiogenic growth factor secretion in ECs (Ucuzian, *et al.*, 2010), whereas HG-induced ROS overproduction interrupts the process of pro-angiogenic growth factor stimulation and secretion. Since the defect of pro-angiogenic growth factor secretion by HG-induced ROS overproduction also influences angiogenesis impairment (Shibuya, 2011) The PPE in this study potentially consists of pro-angiogenic growth factors, which potentially stimulate bFGF and EGF secretion in HG (Figure 16). The PPE in this study may also provide the anti-oxidative effect, whereby the inhibition or reduction of HG-induced intracellular ROS may recovery bFGF and EGF secretion. Unfortunately, VEGF could not be detected in the supernatant. PPE may consist of a few activators of VEGF gene transcription or may not directly affect pro-angiogenic growth factor secretion. However, the determination of intracellular protein and gene expression of pro-angiogenic growth factors may be considered in the future.

In this study, the determining of ECs viability in Figure 11 by using MTT viability assay maybe not appropriate for interpretation. Since the ECs viability showed the minimal difference between HG in the presence of PPE and HG alone, the annexin V/PI staining was also conducted to elucidate the stimulatory effect of PPE on HG-induced ECs viable impairment. The results of the annexin V/PI staining clearly illustrated that HG alone significantly reduced ECs viability, whereby HG alone also significantly increased total apoptotic cells almost twofold. Interestingly, HG in the presence of PPE gradually illustrated the proportional increment of ECs viability depending on the dose compared to HG alone, whereby HG in the presence of PPE also significantly decreased total apoptotic cells compared to HG alone. Especially, HG in the presence of PPE 15 µg/mL significantly reduced total apoptotic

cells almost twofold. In summary, PPE treatment in HG truly improves HG-induced ECs viable impairment by inhibiting ECs apoptosis (Figure 12). The band intensity of phospho-ERK1/2 in NG lane presenting higher than total ERK1/2 in NG lane may lead to the fault interpretation. The process of western blot analysis in this study was rechecked and clarified that was not a consequence of human error or membrane stripping/reprobing. However, the investigation of the biological event of EA.hy926 cells on phospho-ERK1/2 activation may need to fulfill this gap in the future.

The limitation of this study is the use of an *in vitro* model for investigating HG-induced angiogenesis impairment. The *in vivo* model of angiogenesis may mimic the organism, which presents a complete and fully functional angiogenic process. However, the determination of angiogenesis by using the *in vitro* tube formation assay provides reproducibility rather than other the *in vivo* model (Xie, *et al.*, 2016). Remarkably, this is the first study that elucidates the stimulatory effect of PPE on HG-induced angiogenesis impairment and its molecular mechanisms. Since PPE greatly comprises the abundance of growth factors, nutrients, antioxidants, etc., the purification into one agent may debase its integrated bioactive functions. Therefore, the integrated PPE is emphasized rather than a single agent in our study. The investigation of PPE's stimulatory effect on angiogenesis and wound healing in the animal model may require before clinical application. Therefore, future work should focus on angiogenesis and wound healing in a diabetic rat model. The wound biopsy should be stained with CD31 for angiogenesis evaluation in a wound area. The wound tissue should be also homogenized to determine the pro-survival signaling pathway and the apoptotic regulatory molecules.

In summary, PPE ameliorated HG-induced excessive ROS-mediated ECs viable, migrative, and angiogenesis impairment by showing the recovery of PI3K/Akt/ERK1/2 activation. The inhibition of PPE on HG-induced ROS overproduction also attenuated ECs apoptosis by showing the declination of p53, Bax, cleaved caspase 9, cleaved caspase 3 levels, and the increment of Bcl 2 level. Since angiogenesis impairment is one pivotal factor of delayed wound healing in poorly controlled diabetic patients, whereby the risk of amputation is higher. The topical PPE treatment on the wound site may provide a better outcome of wound repair including the preventive potential of amputation in diabetic patients.

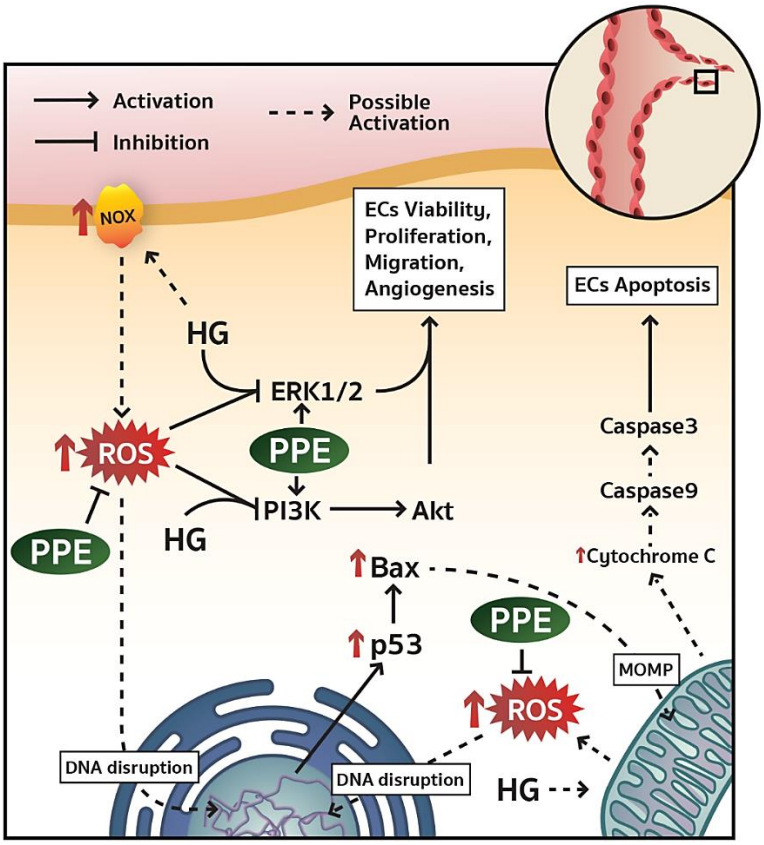

Figure 20 The mechanistic diagram of the potential molecular mechanism of PPE on HG-induced angiogenesis impairment.

PPE inhibits HG-induced intracellular ROS overproduction, whereby it preserved endothelial cell viability, migration, angiogenesis, and also attenuated endothelial apoptosis by showing the recovery of PI3K/Akt/ERK1/2 activation and the rebalance of apoptotic regulatory molecules.

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APPENDIXES

Cell culture reagents' preparation

1. DMEM complete medium for EA.hy926 culture (1 L)
    - DMEM medium base (Thermo Fisher Scientific, MA, USA) 940 mL
    - Fetal bovine serum (FBS) 50 mL
    - Antibiotic/Antimycotic (Thermo Fisher Scientific, MA, USA) 10 mL
    - $NaHCO_3$ 3.7 g

2. Primary vascular cells complete medium for HUVECs culture (500 mL)
    - Vascular cell basal medium (ATCC® PCS-100-030™) (ATCC, VA, USA) 475 mL
    - Endothelial cell growth kit-BBE (ATCC® PCS-100-040™)(ATCC, VA, USA)
        - Bovine brain extract (BBE) 1 mL
        - Recombinant EGF 0.5 mL
        - L-glutamine 25 mL
        - Heparin sulfate 0.5 mL
        - Hydrocortisone hemisuccinate 0.5 mL
        - Fetal bovine serum (FBS) 10 mL
        - Ascorbic acid 0.5 mL
    - Pennicillin-Streptomycin-Amphotericin B solution (ATCC, VA, USA) 0.5 mL

3. FBS-depleted DMEM medium (0.5% FBS) (50 mL)
    - DMEM complete medium 0.25 mL
    - Serum free DMEM medium 49.75 mL

4. Phosphate Buffer Saline (PBS) (100 mL)
   - PBS tablet (AMRESCO, OH, USA) 1 tablet

     Each tablet contains
     - NaCl 4 g
     - KCl 0.1 g
     - Na_2PO_4 0.72 g
     - KH_2PO_4 0.12 g
   - Deionized water 100 mL

Cell lysis buffer

NP-40 lysis buffer (50 mL)
- Tris base 3.03 g
- NaCl 1.47 g
- EDTA 9.306 g
- 10% NP-40 5 µL
- Deionized water 50 mL

  (Adjust the mixed solution to pH 7.4)

SDS-PAGE gel solution

Polyacrylamide gel solution

Polyacrylamide gels were constituted based on the following concentration.

Reagents	**Stacking gel**	**Separating gel**		
	4%	**10%**	**12%**	**X%**
Deionized water	3.075 mL	6.03 mL	5.03 mL	11.03-(0.5xx) mL
30% Acrylamide/bis	640 µL	5 mL	6 mL	0.5xx mL
0.5 M Tris-HCl, pH 6.8	1.67 mL	-	-	-
1.5 M Tris-HCl, pH 8.8	-	3.75 mL	3.75 mL	3.75 mL
10% SDS	50 µL	150 µL	150 µL	150 µL
10% AP	40 µL	120 µL	120 µL	120 µL
TEMED	10 µL	20 µL	20 µL	20 µL
Total volume	5 mL	15 mL	15 mL	15 mL

Western blot analysis's solution

1. Gel running buffer
   - Gel running buffer (10X) contains 250 mM Tris, 1.92 M glycine, 1% SDS and adjusts pH to 8.3
     - Tris 30.30 g
     - Glycine 144.10 g
     - SDS 10 g
     - Deionized water 1 L
   - Gel running buffer (1X) (1 L)
     - Gel running buffer (10X) 100 mL
     - Deionized water 900 mL

2. Gel transfer buffer
   - Gel transfer buffer (10X) contains 25 mM Tris and 190 mM glycine
     - Tris 30.3 g
     - Glycine 144.2 g
     - Deionized water 1 L
   - Gel transfer buffer (1X) (1 L) contains 20% methanol
     - Gel transfer buffer (10X) 100 mL
     - Methanol 200 mL
     - Deionized water 700 mL

3. TBST buffer
   - TBST buffer (10X) contains 20 mM Tris, 150 mM NaCl, and adjusts pH to 7.5
     - Tris 24.2 g
     - NaCl 80 g
   - TBST buffer (1X) (1 L) contains 0.1% Tween 20
     - TBST buffer (10X) 100 mL
     - Tween 20 1 mL
     - Deionized water 900 mL

4. Blocking buffer (5% skim milk) (100 mL)
   - TBST buffer (1X) 100 mL
   - Skim milk powder 5 g

5. Antibody diluent (1% Skim milk in 1XTBST buffer)(100 mL)
   - TBST buffer (1X) 100 mL
   - Skim milk powder 1 g

Approval for research conduction

ประกาศบัณฑิตวิทยาลัย มหาวิทยาลัยนเรศวร
เรื่อง อนุมัติให้นิสิตระดับปริญญาโทดำเนินการทำวิจัย
ครั้งที่ ๕๔/๒๕๖๓

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บัณฑิตวิทยาลัย อนุมัติให้ **นายฉัตรชัย เณรสาตร์** รหัสประจำตัว ๖๑๐๖๐๗๒๐ นิสิตระดับปริญญาโท หลักสูตรวิทยาศาสตรมหาบัณฑิต สาขาวิชาชีวเวชศาสตร์ ดำเนินการทำวิจัยตามโครงร่างวิทยานิพนธ์ที่เสนอ

เรื่อง **ภาษาไทย** "ผลของสารสกัดจากรกหมูต่อการกระตุ้นการสร้างหลอดเลือดฝอยใหม่ในสภาวะน้ำตาลสูง"

ภาษาอังกฤษ "THE STIMULATORY EFFECT OF PORCINE PLACENTA EXTRACT ON *in vitro* HIGH GLUCOSE-INDUCED ANGIOGENESIS DISTURBANCE"

โดยมี ผู้ช่วยศาสตราจารย์ ดร.อรัญญา จิระวิริยะกุล เป็นประธานที่ปรึกษาวิทยานิพนธ์

จึงประกาศมาให้ทราบโดยทั่วกัน

ประกาศ ณ วันที่ ๒๐ เมษายน พ.ศ. ๒๕๖๓

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