Study diabetes-related endothelial cell dysfunction in a hemodynamic microfluidic system

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**STUDY DIABETES-RELATED ENDOTHELIAL CELL
DYSFUNCTION IN A HEMODYNAMIC MICROFLUIDIC SYSTEM**

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STUDY DIABETES-RELATED ENDOTHELIAL CELL DYSFUNCTION IN A HEMODYNAMIC MICROFLUIDIC SYSTEM

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Abstract

Diabetes mellitus is a common metabolic disease with worldwide increasingly high morbidity and mortality, which is a serious threat to human health. It can be classified into two types based on the response to insulin in human body. Type 1 diabetes, of which the patients' pancreas cannot produce enough insulin, accounts for 10% of total amount of patients with diabetes. Type 2 diabetes, of which the cells of the patients cannot respond to insulin properly, makes up 90% of the total cases. The deficiency of blood glucose control in type 2 diabetes significantly increases the risk of long-term complications related to damage to blood vessels, which leads to the major mortality. Hyperglycemia has been proved to be associated with the development and progression of diabetic complications, but it cannot dramatically alter endothelial cell function independently. In our previous study, pulsatile shear stresses along the endothelium, which are the frictional forces caused by blood flow, were demonstrated to contribute significantly to affect vascular functions, which can induce ROS generation and mitochondria fission in endothelial cells.

In this thesis, I have introduced a novel hemodynamic microfluidic system to investigate the hyperglycemia and pulsatile shear stress induced endothelial cell apoptosis. This system was developed to accurately simulate the pulsatile nature of the blood flow in a human artery. Apoptosis was determined by using endothelial cells expressing a fluorescent resonance energy transfer-based biosensor as well as double staining method and Hoechst staining under different levels of glucose and pulsatile shear stresses.

Furthermore, plasma samples from healthy volunteers and patients with diabetes were compared to identify biological factors that are critical to endothelial disruption. Three types of microchannels were designed to simulate the blood vessels under healthy and partially blocked pathological conditions. Results showed that endothelial cell apoptosis rates increased with increasing glucose concentration and levels of shear stress. The rates of apoptosis further increased for 1.4 to 2.3-fold when hyperglycemic plasma was used in the experiments under all hemodynamic conditions. Under static conditions, little difference was detected in the rate of endothelial cell apoptosis between experiments using plasma from patients with diabetes and glucose medium, suggesting that the effects of hyperglycaemia and biological factors on the induction of endothelial cell apoptosis are all shear flow-dependent. A proteomics study was then conducted to identify biological factors, demonstrating that the levels of eight proteins, including haptoglobin and clusterin, were significantly down-regulated, while six proteins, including apolipoprotein C-III, were significantly up-regulated in the plasma of patients with diabetes compared to healthy volunteers. A further study was conducted to investigate the relationship between haptoglobin levels and demographics of patients with diabetes, which showed that the duration of diabetes may potentially associate with the risk of vascular complications.

In conclusion, this dissertation introduces a hemodynamic microfluidic system to investigate the effect of hyperglycemia, pulsatile shear stress and plasma proteins on endothelial cell dysfunction and elucidates that pulsatile nature of blood flow is an indispensable factor of inducing endothelium damage. Last but not least, haptoglobin is a

potential indicator for evaluating the risk of vascular complications in patients with diabetes.

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List of Abbreviations

BMI	Body mass index
CVD	Cardiovascular disease
DM	Diabetes mellitus
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
G6P	Glucose-6-phosphate
Hb	Hemoglobin
HbA1c	Glycated hemoglobin A1c
HDL	High density lipoprotein
HK	Hexokinase
Hp	Haptoglobin
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
LDL	Low density lipoprotein
NADPH	Nicotinamide adenine dinucleotide phosphate with hydrogen
NIDDM	Non-insulin-dependent diabetes mellitus
PPP	Pentose phosphate pathway
R5P	Ribose-5-phosphate
ROS	Reactive oxygen species
SD	Standard deviation

SOD	Superoxide dismutase
TC	Total cholesterol

Chapter 1

Introduction

1.1 Introduction of diabetes

Diabetes mellitus (DM) is a global chronic disease that occurs either when the pancreas beta cells do not produce enough insulin or when the body cannot effectively utilize the insulin it produces to maintain the level of blood glucose. There are two types of diabetes shown in Table 1.1. Type 1 diabetes is immune-mediated that requires administration of insulin, while the other one is type 2 diabetes and characterized by insulin resistance or deficiency of relative insulin. Type 2 diabetes is the most common form and accounts for around 90% of people with diabetes globally. The prevalence of type 2 diabetes increases continually which also increases the numbers of patients at the risk of various diabetic complications. Type 2 diabetes also increases the risk of a myocardial infarction for two times and the risk of suffering heart stroke for two to four times. It is also a leading cause of blindness, kidney failure, and limb amputation [1,2]. Although secondary preventions after myocardial infarction have shown good short-term effect in patients with diabetes, they still cannot obtain a similar reduction in longer-term fatality rates of cardiovascular disease (CVD) compared to the patients without diabetes [3]. Population based studies of the risk factor trends of vascular diseases among patients with and without diabetes show different results in disfavour of those with diabetes [4]. Previous studies also showed that appropriate drug treatment assisted by lifestyle intervention can be effective in delaying or preventing both diabetes and its complications [5-8]. Accordingly, simple and acceptable clinical tools for the identification of diabetic subjects and the relative risk are necessary.

Table 1.1 Characteristics of type 1 and type 2 diabetes

	Type 1 diabetes	Type 2 diabetes
Onset	Sudden	Gradual
Patient distribution	Mostly in children	Mostly in adults
Prevalence	~10%	~90%
Causes	Inherited or certain infection	Lifestyle factors and genetics
Pathogenesis	Unable to produce insulin	Insulin resistance
Therapy	Insulin injection	Exercise, dietary modification, oral drug

1.1.1 Global incidences

The global prevalence of diabetes in 2010 among people aged from 20 to 79 years is estimated to 6.4%, affecting 285 million adults [9]. There is an expected more than 60% increase in numbers of adults with diabetes in developing countries, and more than 15% increase in developed countries between 2010 and 2030 [9]. In Singapore, one out of 9 people aged 18 to 69 has diabetes, which is about 11.3% of the total population or more than 400,000 people suffering. Statistics have shown that there have been more than 3.96 million people die from diabetes and its complications. This number is anticipated to increase by 50% over next decade [10]. Many studies have elucidated that a variety of features, such as high blood glucose (hyperglycemia), dyslipidemia (high triglyceride and total cholesterol levels), obesity, low high density lipoprotein (HDL) cholesterol levels and hypertension, referred to as insulin resistance or the metabolic syndrome, are crucial factors contributing to the increased risk of developing type 2 diabetes as well as for its vascular complications [11,12]. Lifestyle and environmental factors should be the main causes of the increase of type 2

diabetes cases [13-15]. Investigations at genetic level can probably help identifying the most vulnerable to these changes. Furthermore, studies have shown certain ethnic groups may be more liable to develop diabetes [16-19].

1.1.2 Aetiology of type 2 diabetes

Type 2 diabetes is the result of an imbalance between insulin consumption and secretion. Previous studies have indicated that the early abnormality in type 2 diabetes is an injury in the body's ability to respond to insulin at cellular levels. The abnormal insulin action can be observed in many tissues, such as adipose tissue, skeletal muscle, and the liver. These incidences can lead to elevated insulin secretion from the pancreas beta cells to recuperate impaired insulin action. Compensatory hyperinsulinemia can maintain glucose level within healthy range. However, the functions of pancreatic beta cells may gradually decline, which eventually leads to the deficiency of glucose tolerance and the occurrence of diabetes mellitus [20-22].

1.1.3 Risk factors for type 2 diabetes

Previously, many studies have elucidated the relationships between different risk factors and the development of type 2 diabetes. Body mass index (BMI), lipids, hypertension, smoking, physical inactivity, insufficient education, dietary patterns, and also some specific genes are the most commonly mentioned risk factors for the development of type 2 diabetes [23-29].

BMI

Clinical studies have shown that abnormally high BMI is a significant risk factor for type 2 diabetes [24,30-33]. The proportional relationship between body weight and type 2 diabetes can be found both in male [30,33-35], and female [24,30,33,36]. It has been discovered that the adipose tissue can release many types of chemicals that involved in insulin resistance in obese individuals, such as hormones, non-esterified fatty acids, and pro-inflammatory cytokines. When the dysfunction of pancreatic beta cells also occurs, blood glucose cannot be effectively controlled by the reduced level of insulin, thus leading to type 2 diabetes. Some genes that are relative to obesity can interact with environment and lifestyles and lead to diabetes. They were proved to explain several hundred grams differences in body weight and participate in determining BMI [37]. It is also reported that genes responding to insulin resistance can interact with other lifestyle factors including increased fat/calorie intake, as a consequence of which obesity and insulin resistance occur accompanied by type 2 diabetes [38].

Lipids

Many studies have elucidated that the unfavourable blood lipids is an important risk factor for the development of type 2 diabetes [24,25,30,32,33,39]. The potential risk of type 2 diabetes and the level of high density lipoprotein (HDL) has been proved to have an inverse relationship in many case [32,39]. Studies also found that low HDL level would be a more serious risk factor of type 2 diabetes in women [40]. Increased level of triglycerides has been found to be an independent risk factor of type 2 diabetes [33]. In pre-diabetic states, both

low HDL levels and high triglycerides level can be observed with the syndrome of insulin resistance [11,12], indicating that these characteristics can reflect the degree of insulin resistance. Increased levels of free fatty acids in the plasma can induce the elevation of insulin levels and chylomicron's assembly in the gut, as a result of which localized insulin resistance occurs in the intestine. Previous studies have also reported that BMI is related to low levels of high density lipoprotein and high concentration of triglycerides in the plasma [41-43], and BMI can be changed over time with lifestyle interventions, to be proportionally related to the levels of low density lipoprotein, total cholesterol, and triglycerides [44,45]. All the plasma lipids have been proved to associate with diabetes mellitus independently except triglycerides.

Hypertension

Previous clinical studies have elucidated that hypertension is a crucial factor of type 2 diabetes [31,46-48]. Many factors should be the causes that involve in the association between hypertension and type 2 diabetes. The most prominent one of pathophysiological causes could be endothelial dysfunction. Many studies have clarified the markers of endothelium dysfunction in the development of diabetes [49,50], as well as their relationships to the adjustment of blood pressure [51]. Some markers in inflammatory reactions, such as C-reactive protein, are consistently related to type 2 diabetes [52], and the elevation of blood pressure [53], indicating that inflammation should be a potential association of blood pressure and type 2 diabetes [54]. Moreover, insulin resistance is also relative to blood pressure levels in type 2 diabetes [55]. In addition, reports from clinical

studies have suggested the correlations between blood pressure, body weight, and the incidences of type 2 diabetes [42-44,56]. Although it is proved that blood pressure can increase proportionally with BMI, the association between hypertension and type 2 diabetes is still independent of the change of BMI. A randomized clinical trial have shown a 14% decrease of risk of diabetes in patients with glucose intolerance by treatment with angiotensin II blocker valsartan for five years, which further strengthens the relationship between hypertension and type 2 diabetes [57].

Smoking

Previous investigations have indicated that smoking is also an outstanding factor for the development of type 2 diabetes [58-61]. A recent meta-analysis containing 25 studies elucidated that smoking was related with a 44% elevated risk of diabetes [62]. The relationship became much more obvious in heavy smokers who took more than 20 cigarettes per day compared with light or former ones [62-64]. Additionally, there are some studies elucidating that the smokers will obtain a higher risk of type 2 diabetes in their first two or three years of cessation [58,59]. Smoking is proved to be associated with insulin resistance and inadequate compensatory insulin secretion response [65-68], which is caused by the effect of nicotinic and other toxic components in cigarette on pancreatic beta cells. It was further suggested to have a relationship with chronic pancreatitis and pancreatic cancer [69]. Furthermore, heavy smokers were indicated to have higher risks of occurring type 2 diabetes, with an increased systemic inflammation that obtain significant increase of the body weight

after cessation [70]. A long-term clinical investigation showed a decrease in risk of obtaining type 2 diabetes after smoking cessation [71].

Physical inactivity

Population based studies have indicated that lack of physical inactivity is an essential risk factor for the development of type 2 diabetes [72-75]. It was reported that prolonged television watching would be a prominent characteristic of sedentary lifestyle that positively related to the risk of diabetes in human [76-78]. People lived with adequate physical activity had a lower risk of developing type 2 diabetes [72,79]. Clinical trials have provided evidence that type 2 diabetes could be delayed or even prevented as a result of successful and effective lifestyle interventions which included physical activity as an integral part [5,80]. Furthermore, physical activity also plays an indispensable role in directly enhancing insulin sensitivity and declining insulin resistance as well as indirectly maintaining body weight, thus delaying or even preventing the development of type 2 diabetes [81-83].

Insufficient education

Some population based investigations have reviewed the relationship between the incidences of diabetes and educational levels of individuals. It was revealed that low education could be a noticeable factor of type 2 diabetes, differing from other biological factors [23,84,85]. A previous study have reported that people with a degree lower than high school diploma would have a twice higher possibility to have diabetes, compared to those with a bachelor degree or higher [86]. Another study provided evidence that women with lower education

may have a higher risk of obtaining diabetes than those educated better. Moreover, this relationship differed from races, ethnicities and gender [87]. Another recent investigation revealed that the risk of developing type 2 diabetes was higher in the less educated individuals who were also obese, compare to the better educated ones [88]. All these studies demonstrate that educational level can provide information and promote interests of obtaining a healthy life that significantly influence people's attitude to reduce the risk of developing diabetes.

Dietary pattern

Dietary pattern is another important lifestyle factor that related to the development of type 2 diabetes. It was reported that different patterns of food consumption can be associated with the risk of type 2 diabetes [89-92]. The elevated risk of type 2 diabetes was proved to be related to higher dietary glycemic index in clinical studies [92,93]. The relative risk of type 2 diabetes from the highest to the lowest glycemic index was 1, 1.15, 1.07, 1.27, and 1.59 respectively ($P \leq 0.001$). Comparatively, the consumption of cereal fiber intake related to a decreased risk of diabetes [92]. Furthermore, a study on the consumption of rice has revealed that people who take brown rice or other whole grains in replacement of white rice have a lower risk of developing type 2 diabetes [90]. In a review which have concluded from 19 studies, it is stated that the increased consumption of long-chain n.3 fatty acid and polyunsaturated fat is favorable, while the increased consumption of trans fat and saturated fat can negatively affects glucose metabolism and insulin action [94]. Another study has found that higher intake of potatoes, butter, and whole milk was related to the increased risk

of type 2 diabetes, while higher intake of fruits and vegetables can help reducing the risk of type 2 diabetes [95]. The underlying mechanisms of these studies are that the consumption of insoluble fiber should be consistently linked to the improvement of insulin action and the reduction of the risk of developing type 2 diabetes [96,97]. Long-term observation based studies have demonstrated that the *in vivo* status and the intake of vitamin D were proportionally associated with the incidences of type 2 diabetes [98,99], suggesting that the deficiency of vitamin D can contribute to the dysfunction of pancreatic beta cells and insulin resistance and result in type 2 diabetes. The influence of dietary patterns in the above studies has been proved to be independent of BMI changes.

Genetics

Many studies have indicated that genetic factors play an indispensable role in the pathogenesis of type 2 diabetes [13,100-102]. Some prospective studies have demonstrated that the diabetic history among the first degree relatives of family members suggests a higher risk of type 2 diabetes, and the risk is even greater when both parents are affected [100,101,103,104]. Another study on twins have elucidated that the concordance estimate of the development of type 2 diabetes is higher in monozygotic twins than in dizygotic [105]. Furthermore, prevalence of diabetes varies significantly in different races [13]. This investigation of substantial variation in the prevalence of diabetes was conducted across the ethnic groups that share a similar living environment, suggesting the contribution of genetic factors on disease predisposition [106]. Research works have also supported that some genes predispose to developing type 2 diabetes by affecting insulin action and secretion in most of

human cases [106-108]. Researchers have already identified variants in 11 genes, including CDKAL1, FTO, HHEX, IGF2BP2, JAZF1, KCNJ11, NOTCH2, PPARG, SLC30A8, TCF7L2, and WFS1, to be considerably relative to the risk of type 2 diabetes which is independent of other clinical factors. Eight of these genes were linked to impaired pancreatic beta cell function [29]. The transcription factors 7-like 2 (TCF7L2) is the most prominent one with the highest risk of developing type 2 diabetes among the genes that expressed in pancreatic cells [29,109-111]. Although the relative research methodology is mature and sufficient, the genetic information is still for research purposes only so far.

1.2 Vascular complications of diabetes

Diabetes is usually underreported as a cause of death probably for its leading to many types of complications. Overall, the possibility of death in patients with diabetes is twice as the people of the same age but do not suffer diabetes [112]. Some epidemiological data elucidated that the prevalence of diabetes was dramatically elevating among young adults and pregnant women [113]. Diabetes is a prominent predictor of premature mortality for its association with a substantial increase in total mortality from all diseases [114]. More than 65% patients with diabetes die from cardiovascular disease, in which ischemic heart disease and stroke comprise the largest proportion of morbidity [112]. Furthermore, there is a grimmer tendency in the increase of diabetes incidences in developing countries compared to the well-developed regions, due to the aging problems, complicated demographics, and the increase of urbanisation.

The vascular complications of diabetes have been proved to be the major cause of morbidity and mortality globally, which lead to the increase of cost for healthcare products and medical treatments. Many organs in the body system will be influenced by diabetes, inducing vascular complications that categorized as microvascular and macrovascular. Microvascular complications consists of eye damage (retinopathy), renal system damage (nephropathy), and nervous system damage (neuropathy). Macrovascular complications mainly consists of cardiovascular disease, heart stroke, and atherosclerosis. Peripheral microvascular complications may lead to injuries that are hard to heal, and eventually gangrene or amputation [112]. All these complications are increasing at an alarming rate in the world. Currently, more than 50% of patients with type 2 diabetes have already been found evidence of occurring vascular complications when receiving diagnosis [115].

Vascular complications of diabetes can be either episodic (e.g., foot ulceration) that can be treated or re-occur for numerous times, or progressive (e.g., retinopathy) which normally initiate mildly but develop seriously, resulting in severe damage and irreversible loss of functionality in the organs [116]. There are also some other types of diabetic complications, including the reduction of resistance to infections such as pneumonia and influenza, dental disease, and other diseases that come with birth. Although the types of complications are similar for type 1 and type 2 diabetes patients, the frequency or timing of occurrence can be different [112].

Diabetes can affect human health in many ways. The most disreputable one is the development of various long-term complications. The quality of life will drop dramatically for the patients with diabetes, along for chronic pain, heart problems, visual deficiency, renal

diseases, and even amputations [117,118]. Meanwhile, both patients and society have to consider the economical and mental cost for the therapy of diabetes. Currently, cardiovascular diseases are the major lethal cause for patients with type 2 diabetes. Diabetic retinopathy accounts for about 5% of all blindness cases all over the world. More than half of patients with diabetic nephropathy are receiving renal replacement therapy (RRT) [115]. Furthermore, there are still some unidentified or uncontrolled patients with diabetes, resulting in life-threatening long-term complications and even disabling [119].

Despite the fact that there has been reported an improvements in the control of blood glucose in adult patients with diabetes, less than 15% of the treatment outcomes could successfully achieve three other therapeutic standards simultaneously, including blood glucose, blood pressure and concentration of low-density lipoproteins (LDL) in the blood [120]. Some early studies have demonstrated that optimal glycaemic control is a primary preventive measure against the development of diabetic vascular complications [121].

1.3 Glucose metabolism

Glucose is the key energy source for the cells as they can be used by the cells to produce ATP *via* glycolysis metabolic pathway. The first step of the glucose metabolism after its entering into the cell is the phosphorylation of glucose to form glucose 6-phosphate (G6P) by hexokinase (HK) which can prevent glucose diffusing out of the cell (Figure 1.1) [122]. The activation of HK can be regulated by glucose herein extracellular glucose firstly induces insulin and insulin-like growth factor 1 (IGF1) secretion into the bloodstream. Insulin and

IGF1 can bind and activate insulin receptor (IR) and insulin-like growth factor receptor (IGFR) to further activate PI3K and Akt resulting in HK activation.

G6P can be further metabolized through two different pathways: glycolysis and pentose phosphate pathway (PPP). Glycolysis is the major linear pathway which converts glucose to pyruvate. Pyruvate can be further oxidised by the Krebs cycle in the mitochondria to support effective oxidative phosphorylation which generates the majority of the cell's ATP, or reduced to lactate in the cytosol (Figure 1.1) [122]. PPP is a minor pathway which diverts G6P into ribose-5-phosphate (R5P). During this process, antioxidant NADPH was generated which regulates the redox state of the cell. The abnormal elevation of glucose will result in higher uptake of glucose, heavy loading of the glycolysis pathway, bypassing of the PPP, which leads to the imbalance of redox state of the cell.

Figure 1.1 Glucose metabolism in the cells. The figure is reprinted from [122] with permission.

Although cells can be powered by the ATP molecules generated during glycolysis, this process can produce significant amount of reactive oxygen species (ROS). ROS are

chemically reactive molecules containing active oxygen. They are natural by-products of the normal aerobic metabolism in cells, which include superoxide anions (O_2^-), hydroxyl radicals ($-OH$) and hydrogen peroxide molecules (H_2O_2) [123]. ROS are highly reactive due to the presence of unpaired valence shell electrons. Although normal level of ROS has important roles in cell signalling and homeostasis [124], abnormal high level of ROS can cause oxidative stress to the cells and even induce cell death [122]. Thus programmed cell death may be evolved from the cellular response to metabolic stress, most notably in relation to glucose metabolism (Figure 1.2) [122]. In this theory, the ROS produced during glycolysis in the mitochondria can be a critical activator of different forms of cell death including necrosis, autophagy and apoptosis. Releasing of cytochrome c (Cyt c) from mitochondria to cytosol can activate apoptosis which makes it a key pro-apoptotic protein. Although PPP is a minor pathway of glucose metabolism, the production of antioxidant NADPH can be crucial for cell survival (Figure 1.2) [122].

Figure 1.2 Pathways linking glucose metabolism and cell death. This figure is reprinted from [122] with permission.

Cells have developed complicated system to maintain the level of ROS so that its potential damaging effects can be avoided. For example, cells use antioxidant enzymes such as superoxide dismutase (SOD) to convert O_2^- to H_2O_2 which can be further converted into H_2O and O_2 by catalase and peroxidase. In addition, cells use antioxidant agents such as glutathione and NADPH to scavenge ROS. By using this antioxidant system, the level of ROS can be maintained at a low level in normal cells. However, in patients with diseases such as diabetes mellitus, the levels of ROS can be elevated dramatically, thus causing significant damages to the cells [123].

In particular, this increased level of ROS can result in the oxidative stress in important cells in patient's body and further leads to inflammation, fibrosis and endothelial dysfunction [124]. The process of ROS generation implicates many enzymatic systems, including mitochondrial respiration, uncoupled endothelial nitric oxide synthesis and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase, among which NADPH oxidase appears to be a particularly important factor [125,126]. Since changes in cellular function resulting in oxidative stress play an important role in the development and progression of diabetic micro-vascular diseases, interventions to reduce ROS production by using antioxidant or targeting NADPH oxidase appears to be an attractive therapeutic strategy [127].

In patients with diabetes, the increase in ROS levels causes oxidative stress on endothelial cells, which leads to endothelial cell dysfunction. Those damaged endothelial cells can be recognized by the body, and to be destroyed by a process of programmed cell death, apoptosis [128]. The most common mechanism of apoptosis activation in cells is *via* the caspase pathway [129].

1.4 Study ROS generation and mitochondria fission in endothelial cells under simulated hemodynamic and diabetic conditions

The endothelium is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels, forming an interface between circulating blood and lymph in the lumen and the rest of the vessel wall (Figure 1.3) [130]. The cells that form the endothelium are known as endothelial cells. Endothelial cells in direct contact with blood are called vascular

endothelial cells whereas those in direct contact with lymph are known as lymphatic endothelial cells.

Figure 1.3 Cross-sectional view of a blood vessel. The figure is reprinted from [130] with permission.

Vascular endothelial cells line the entire circulatory system, from the heart to the smallest capillaries. These cells have very distinct and unique functions that are paramount to vascular biology. Endothelial cells function as a selective barrier between the vessel lumen and surrounding tissues, controlling the passage of materials and the transit of white blood cells into and out of the bloodstream [131].

Under normal conditions, endothelial cells synthesize important bioactive substances such as nitric oxide that regulate healthy blood vessel function and structure. Nitric oxide is responsible for the dilation of blood vessels and mediates the endothelium's control of vascular relaxation. Furthermore, nitric oxide plays an important role in the prevention of

atherogenesis by inhibiting platelet activation and limiting inflammation by reducing leukocyte adhesion to the endothelium [132].

In patients with diabetes, a number of fundamental mechanisms result in the decreased bioavailability of endothelium-derived nitric oxide. In diabetes, a combination of hyperglycemia, excess free fatty acid release, and insulin resistance results in a cascade of metabolic events within the endothelial cells which decreases the production of nitric oxide. The decrease in nitric oxide leads to an increase in production of ROS in endothelial and vascular smooth muscle cells [133]. The increase in ROS levels leads to endothelial dysfunction, augments vasoconstriction, increases inflammation, and promotes thrombosis, which in turn results in plaque formation in blood vessels (atherogenesis), leading to atherosclerosis and other cardiovascular diseases. Despite the fact that there are many studies investigating the increase of ROS level in endothelial cells [134-137], few of them had considered the hemorheologic and dynamic conditions in the blood stream along the endothelium. Accordingly, a necessity underlies the research on endothelial cell functions under pulsatile nature.

Our research group is the first one to apply a hemodynamic microfluidic system to study the cellular responses of endothelial cells under a physiological pulsatile shear force-induced condition [125]. The most important advantage of our system over traditional methodology which studies cells under static condition is that we studied cell behaviors and functions under fluidic conditions simulating the pulsatile blood flow in a human artery. As shown in Figure 1.4, this microfluidic networks consists of two injection inlets which can supply a concentration gradient of glucose to three branches of microchannels. Each branch of the

microchannel has 4 cell culture sites to perform a series of time course experiments. Each cell culture site has a width of 600 μm and a height of 150 μm to mimic the small arteries [125].

(a)

(b)

Figure 1.4 Schematic illustrations of (a) the design of the microfluidic chip, and (b) the hemodynamic lab-on-a-chip system with a configurable flow profile to mimic the blood flow in a blood vessel. This figure is reprinted from [125] with permission.

Endothelial cells were then injected into the microchannels at the concentration of about 1×10^7 cells/ml. The microfluidic chips were supplied with culture medium and replaced with fresh medium at every 24 hours until the cells in the microchannel reached almost full confluence for the experiments. Figure 1.5 shows the endothelial cells at the cell culture site in the chips after initial seeding and after 48 hours culture in the microchannel with a well formed monolayer of cells.

Figure 1.5 Phase images of endothelial cells (a) seeded in the microchannel and (b) cultured in the microchannel for 48 hours. The figure is reprinted from [125] with permission.

ROS levels at static and dynamic conditions under high glucose level exposure were then measured by pre-staining cells with a fluorescent dye, $10 \mu\text{M}$ H_2DCFDA , for 30 min. A pulsation free pump was used to provide constant flow and pulsatile flow along the endothelial monolayer. The normalized temporal responses of the endothelial cells in ROS level under different flow conditions are shown in Figure 1.6 [125]. The static condition without a fluidic flow serves as a negative control of the experiment. Figure 1.7a shows the intracellular ROS level after exposing the endothelial cells to pulsatile shear stress at 30 dyne/cm^2 for different time durations. Within 60 min, the ROS level was gradually increased

along the time, but prolonged exposure of the endothelial cells to the pulsatile flow up to 2 hours resulted in a sustained elevation in ROS levels (Figure 1.6). The ROS levels of endothelial cells under different shear treatment conditions for up to 120 min are shown in Figure 1.7b [125]. At the 60 min time point, there is no significant difference of ROS level between the treatment of 30 dyne/cm² constant flow and the static condition. Oppositely, the increment was outstanding when the endothelial cells were being exposed to physiological pulsatile shear stress conditions. The ROS level was increased by 1.7 fold and 2.2 fold at pulsatile shear stress of 15 and 30 dyne/cm², respectively (Figure 1.6). This results show that it is necessary to investigate the effect of shear stress on the endothelial cells under a physiological condition, which can accurately reflect the pulsatile pattern of the blood flow in the artery. Additionally, reports stating that pulsatile shear stress is critical to maintain the functionalities of endothelial cells in the artery have been presented [138,139]. The pulsatile nature should be important to ROS generation in endothelial cells.

Figure 1.6 Temporal responses of the ROS level in endothelial cells under different shear stress profiles. This figure is reprinted from [125] with permission.

Figure 1.7 The ROS level in endothelial cells being exposed to (a) a pulsatile shear stress of 30 dyne/cm² for different times and (b) different shear treatment conditions for 60 min. The figure is reprinted from [125] with permission.

Afterwards, the researchers have also investigated the mitochondrial morphology of endothelial cells being exposed under different shear treatment conditions using MitoTracker staining and the fluorescent images are shown in Figure 1.8 [125]. Under static condition, only 2% of the cells had shorter and smaller mitochondria, indicating

mitochondrial fragmentation. When the endothelial cells were exposed to a physiological pulsatile shear stress, some cells exhibited mitochondrial morphological changes. As shown in Figure 1.8, during the first 3 hours exposure to pulsatile shear stresses, the mitochondrial fission percentage of the endothelial cells increased along the time. Higher shear stresses induced higher rate of mitochondrial fission to up to 7.6%.

Figure 1.8 Mitochondrial morphology of endothelial cells under different shear treatment conditions for 3 hours. This figure is reprinted from [125] with permission.

In healthy people, the concentration of fasting glucose in the blood is ranging from 4 to 6 mM. However in patients with diabetes, the concentration of glucose in the blood is above 6 mM and can reach up to 20 mM. In the following investigations [125], 10 mM of glucose was added to mimic the plasma condition of a diabetes patient and the ROS level of the endothelial cells was monitored under normal physiological pulsatile shear stress condition or under exhaustive one. As shown in Figure 1.9, when the endothelial cells were being exposed to 10 mM glucose under the normal pulsatile shear stress of 15 dyne/cm², the ROS level was quickly increased for over 4-fold in the first 60 min and stabilized afterward. By comparing the ROS level at the 60 min time point between the cells treated with 10 mM glucose in a static state and that under a normal shear stress of 15 dyne/cm², the ROS level was increased 1.7 fold [125]. Although high concentration of glucose elevated the level of

intracellular ROS, a much higher level of ROS was detected if the glucose solution was delivered to endothelial cells under a physiological pulsatile shear stress condition.

Figure 1.9 The ROS level in endothelial cells being exposed to 10 mM glucose with normal pulsatile shear stress of 15 dyne/cm². The figure was reprinted from [125] with permission.

Figure 1.10 shows the results of endothelial cells being exposed to the exhaustive pulsatile shear stress of 30 dyne/cm² which can be achieved under heavy exercise conditions [125]. Similar to the ROS elevation profile observed under the normal pulsatile shear stress of 15 dyne/cm², the ROS level was significantly increased for nearly 5-fold in the first 60 min and stabilized afterward. By comparing the ROS level at the 60 min time point between the cells treated with 10 mM glucose in a static state and that under the high level of shear stress of 30 dyne/cm², the ROS level was increased 1.9-fold. By comparing the ROS level in the 60 min time point between the one with normal pulsatile shear stress of 15 dyne/cm² and the one with a fast pulsatile shear stress of 30 dyne/cm², the ROS level was elevated by 15%. To sum up, high glucose level under exhaustive exercise condition can substantially increase the intracellular level of ROS in endothelial cells.

Figure 1.10 The ROS level in endothelial cells being exposed to 10 mM glucose with fast pulsatile shear stress of 30 dyne/cm². This figure is reprinted from [125] with permission.

To investigate the mitochondrial morphological changes of endothelial cells under diabetic conditions, 20 mM of glucose was added and the endothelial cells were being exposed under different shear treatment conditions for 4 hours [125]. The fluorescent images of the endothelial cells after being exposed for 4 hours and the mitochondrial fission ratios monitored for the first 4 hours are shown in Figure 1.11. For endothelial cells treated with 20 mM glucose for 4 hours, mitochondrial fragmentation was prevalent with a mitochondrial fission ratio of 20%. When the endothelial cells were being exposed with 20 mM glucose plus the pulsatile shear stress of 15 or 30 dyne/cm², the mitochondrial fission ratio was further elevated from 20% to 25% and 26%, respectively. The results show that hyperglycemia treatment produced a more dominant effect in promoting mitochondrial fission than the physical treatment, especially in such an extreme case of 20 mM of glucose.

Figure 1.11 Mitochondrial morphology of endothelial cells with 20 mM glucose under different shear treatment conditions. The figure is reprinted from [125] with permission.

Once the pulsatile shear stresses exist, the endothelial cells cannot completely avoid ROS generation and downstream oxidative stresses in the blood microenvironment, which may consequently induce endothelium dysfunctions or hemolytic anemia [140]. In human body, haptoglobin, which can bind to and remove the free subunit of hemoglobin, protects and maintains the redox status of blood microenvironment.

1.5 Haptoglobin and diabetes

Haptoglobin is a part of blood plasma proteins. The normal serum concentration of haptoglobin (Hp) ranges between 0.2 and 1.4 mg/ml, and clinically it is a useful marker for several inflammation-related disorders such as hepatitis C, HIV, myocardial infarction, retinal hemorrhage and tuberculosis [141-144]. As a part of essential blood proteins, the haptoglobin in the blood plasma will binds free hemoglobin (Hb) released from red blood cells with high affinity to form stable Hp-Hb complexes and thereby inhibits its oxidative activity [145,146]. The complexes bind to an Hb scavenger receptor CD163 located on the outer envelope of monocyte and macrophages [147]. As a result, they are rapidly removed by the reticuloendothelial system in the liver [148]. The scavenging mechanism is essential in the inhibition of Hb glomerular filtration, which can cause renal failure [149,150]. Hp-Hb complexes also bind directly to receptors on hepatocytes and hepatoma cell lines [151], for internalization into the liver parenchymal cells where organelles carry them into the microsome fraction where they are dissociated and degraded into smaller subunits [152,153]

Hp synthesis is stimulated by several cytokines during inflammation [154]. For instance, IL-6 mediates Hp gene expression and protein synthesis in the liver of most species that have

been studied, including human and rat [155,156]. Hp is initially synthesized as a single chain that is cleaved into two chains; an N-terminal light α -chain (complement-control repeat) and a C-terminal heavy β -chain (serine protease-like domain) [157-159]. The α - and β -chains are covalently attached by an intermolecular disulfide bond made between Cys131 and Cys248 [160]. In most mammals, Hp is formed by two (α - β) monomers linked together covalently by a disulfide bond between the two α -chains creating an (α - β)₂ structure. This form is known as Hp 1-1 in humans (Figure 1.12) [158,159]. However, three different Hp phenotypes are found in humans because of the existence of two Hp gene alleles, known as HP1 and HP2. An intragenic duplication of the HP1 allele forms the HP2 allele, which carries a bigger α -chain with a duplicated cysteine residue linking the α -chains. Consequently, other phenotypes of Hp such as Hp2-1 and Hp2-2 present a range of different HP structures [158,159] as shown in Figure 1.12. Hp 1-1 is a small molecule (86 kDa) with a well-defined linear structure, whereas Hp 2-1 is characterised by heteropolymers (86-300 kDa) and Hp 2-2 forms even bigger macromolecular complexes (170-1000 kDa) [161].

Figure 1.12 Hp structural phenotypes. Schematic drawing showing the molecular arrangement of human Hp phenotypes. The figure is reprinted from [161] with permission.

Besides, iron derived from hemoglobin can catalyse a number of oxidative reactions which can be inhibited by haptoglobin [162]. Firstly, ferrous heme iron (Fe^{2+}) can react with hydrogen peroxide to produce ferric hemoglobin (Fe^{3+}) with by-products of hydroxyl radical species. These highly reactive hydroxyl radicals can initiate the process of lipid peroxidation by extracting a hydrogen atom from poly-unsaturated fatty acids. Secondly, ferrous Hb (Fe^{2+}) can also react with hydrogen peroxide to yield ferryl hemoglobin (Fe^{4+}), which is a highly

unstable molecule that can react with a second molecule of hydrogen peroxide to produce ferric hemoglobin (Fe^{3+}) and superoxide anion. The damage effects of superoxide are in two aspects. It can reduce of ferric iron (Fe^{3+}) in hemoglobin to ferrous iron (Fe^{2+}), following by the production of hydroxyl radical species as described in the first reaction. The dismutation of superoxide anion can produce hydrogen peroxide, then promoting the production of ROS. Finally, ferric hemoglobin which is also known as methaemoglobin, can spontaneously transfer its heme part, resulting in heme entry into diverse lipophilic environments such as low density lipoprotein. Once participated into its new lipid environment, iron in heme will join the reactions with hydrogen peroxide as described above. It can further react with adjacent lipid peroxides, generating a free radical cascade and leading to extensive lipid oxidation.

As a part of the Hp-Hb complex, haptoglobin can stabilize heme in the heme pocket of hemoglobin, and prevent hemoglobin from inducing oxidative damage [163]. However, different types of haptoglobin can neutralize the redox activity of heme iron to different degrees. This has been shown in a variety of research works both *in vitro* and *in vivo*. For instance, studies using linolenic acid elucidated that Hp1-1 can prevent oxidation (diene formation) as measured by an increase in absorbance at 232 nm compared to Hp 2-2 [164]. Another study has investigated the oxidation of low density lipoprotein due to heme transfer from hemoglobin. Heme transfer was measured by quenching of the fluorescence signal emitted by dansylated lipoprotein. It was revealed that Hp1-1 was superior in preventing heme transfer from hemoglobin as compared to Hp2-2 [165].

Studies have also shown that Hp-Hb (2-2) complexes are cleared less efficiently than non Hp-Hb (2-2) complexes [164,165]. In type 2 diabetes, this phenomenon is more pronounced due to the down-regulation of CD163, particularly in Hp2-2 individuals [166]. An impairment in anti-inflammatory macrophage signalling through a CD163/pAkt/IL-10 axis is also seen in Hp2-2 patients [167].

Hp-Hb deficient clearance in Hp2-2 type 2 diabetes individuals results in increased Hp-Hb binding to apolipoprotein A1, a common type of high-density lipoprotein (HDL), thereby tethering the pro-oxidative heme moiety. This renders it deficient in its ability to reverse transfer cholesterol from macrophages [168,169]. The Hp2-2 protein is less efficient at blocking heme transfer from Hb compared to Hp1-1. Furthermore, the increase in heme transfer when Hb is glycosylated may provide a mechanistic explanation for the increase in cardiovascular disease seen in Hp 2-2 type 2 diabetes [170].

Accordingly, deficiency in haptoglobin and Hp2-2 phenotype is associated with decreased ability to bind with Hb, decreased clearance of Hp-Hb (2-2) complexes, impairment in anti-inflammatory signalling pathway, increased LDL oxidation, and less efficient in blocking heme transfer from Hb to Hp1-1. These all lead to a higher risk of cardiovascular diseases. In patients with diabetes, the synergetic effect of hyperglycaemia and haptoglobin phenotype may exacerbate the oxidative stresses, thus contributing to endothelial cell apoptosis.

1.6 FRET-based caspase sensor for detecting endothelial cell apoptosis

Apoptosis can be activated through extrinsic and intrinsic pathways, both of which can activate the most significant execution protease, caspase-3, which can cleave many important structural proteins including actin and nuclear lamin to cause cell destruction and cell death. In this dissertation, most of experiments required a real-time detection of endothelial cell apoptosis in the microfluidic systems as pre-/post-staining of apoptotic markers in the microchannels would dramatically influence the results. Thus a quick measurement of caspase-3 activity in endothelial cells is needed.

To detect the activation of caspase-3 in living cells, a GFP-based fluorescence resonance energy transfer (FRET)-based biosensor was transfected to endothelial cells. The FRET sensor was constructed by fusing a cyan fluorescent protein (CFP) with a yellow fluorescent protein (YFP) with a specialized linker containing the caspase-3 cleavage sequence Asp-Glu-Val-Asp (DEVD) [171]. As shown in Figure 1.13, when the FRET sensor is excited with a light at the excitation wavelength of 433 nm for CFP, the CFP acts as a donor, transferring the energy of emission light *via* FRET to the YFP, which acts as an acceptor. As a result, the majority of the light is emitted from YFP at the wavelength of 526 nm. When cells are undergoing apoptosis and caspase-3 is activated, the DEVD sequence linking the CFP and the YFP would be cleaved. This results in the separation of the donor and the acceptor, effectively eliminating the FRET effect. Therefore, when apoptotic cells are excited again with a light at the excitation wavelength for CFP (433 nm), the majority of light is emitted from CFP at 480 nm. By measuring the fluorescence emission ratio between

YFP and CFP, the activation of caspase-3 can be easily detected in living cells during apoptosis in real-time.

Figure 1.13 Principle of using a FRET based-biosensor to detect caspase-3 activation in apoptotic cells [172]. The figure is reprinted from [172] with permission.

1.7 Significance and overall objectives

The vascular complications of diabetes are a huge health problem which affects 3% of the world population and 11.3% of adults aged between 18 and 69 years in Singapore. These complications can lead to blindness, kidney failure, heart disease and food ulcer resulting in limb amputation. The major cause of vascular complications for diabetes mellitus is now considered to be the elevation of ROS levels in endothelial cells lining blood vessels, which results in oxidative stresses.

There are two factors which may induce this ROS elevation: hyperglycemia and pulsatile shear stress in human blood vessels. And these two factors may reinforce each other. Most existing research investigate hyperglycemia under static condition or using laminar shear stress models with either a constant or cycled flow rate. These models do not simulate the physiological conditions of shear stress in real life situations. Based on our previous studies in which a hemodynamic Lab-on-a-chip system was developed to mimic the pulsatile nature of the blood flow. It was found that the application of a pulsatile flow was the essential factor in accounting for the observed levels of ROS elevation and apoptosis in endothelial cells. In particular, a small increase of ROS was detected in the condition of constant shear stress while more than a 2-fold increase of ROS was detected in cells exposed to a pulsatile shear stress. A much higher level of ROS was detected when different concentrations of glucose were applied to endothelial cells under a pulsatile vs constant shear stress. Thus, it is necessary to introduce the pulsatile shear stress into the model to reflect the fluctuating flow rate of blood in the human body.

In this dissertation, the major objective is to further investigate the endothelial cell dysfunction under pulsatile shear stresses with the novel developed hemodynamic microfluidic system. According to the studies on ROS generation and mitochondria fission, I further explored the apoptosis of endothelial cells under hyperglycemia and pulsatile shear stresses. Additionally, I have applied plasma samples from patients with diabetes to further elucidate what are the crucial plasma components in the blood that relate to endothelial cell apoptosis.

Further investigations were focused on an important plasma protein haptoglobin, of which the major function is to bind to and remove free hemoglobin subunit to reduce hemoglobin-related oxidative stresses. Demographic characters of patients with diabetes were correlated to the haptoglobin expression levels. Meanwhile, plasma of diabetic rat models were used as a comparison to human plasma as this comparative study will exclude the influences of hyperglycemia.

I hope the findings from this thesis can provide proof-of-concept evidence for the pathogenesis of diabetes related complications. The outcomes of this thesis shall provide new insights to the function of antioxidants and haptoglobin on diabetes. Last but not least, I also expect that the hemodynamic microfluidic system can be more widely used in the functional diagnosis of vascular systems in patients with diabetes.

Chapter 2

Materials and methods

2.1 Soft lithography

The microfluidic chip was fabricated in PDMS material using the standard soft lithography process. The microchannel mold was fabricated using SU8-2075 on a silicon wafer with the height of the microstructures at 150 μm , following the steps described in the working flowchart (Figure 2.1). PDMS is transparent, air permeable, bio-compatible, and suitable for making hemodynamic devices.

Figure 2.1 Working flowchart of microchannel mold fabrication, designed for 150 μm height microstructures.

2.2 Hemodynamic lab-on-a-chip system

Two improved hemodynamic lab-on-a-chip system will be shown in the following chapters respectively for different experimental designs. The microfluidic chip was fabricated with polydimethylsiloxane (PDMS) material by the standard soft lithography process. The PDMS

slab was bonded to a glass slide by exposing to air plasma for 15 s using a handheld corona treater (BD-25, Electro-Technic Products). The fabricated PDMS chip was stored for at least 1 day to regain the hydrophobicity of the PDMS microchannel. This step ensures the micro fluid can form a large contact angle with the slides in case of any jam in the middle of the channel.

The microfluidic network consists of several rows of microchannels; and each microchannel has three microchambers, which serves as the cell culture sites. The separated microchannels are connected by flexible connectors. The connection condition and sequence of the channels can be easily adjusted, which facilitate in conducting series operation of fluorescent dye staining and washing and also in performing parallel experiments under different flow conditions. The flow condition was controlled by a pulsation free precision pump (Nemesys, Cetoni), with a flow profile mimicking the blood flow in the blood vessel. The number of microchannel rows can be increased when more complex and varying flow condition processes are needed. By using this microfluidic system, a single microfluidic chip does not only perform a series of experiments with different pulsatile flow conditions simultaneously, but also realizes the parallel comparison among several cell culture chambers within one experimental condition.

2.3 Protocol for culturing endothelial cells

Normal human umbilical vein endothelial cells (HUVEC) cell line CRL1730 was obtained from ATCC, USA. Culture medium was prepared by mixing low glucose (1.0 mg/mL) DMEM (Gibco, USA) with 10% (v/v) fetal bovine serum (FBS, Hyclone, USA) plus 100

units per mL penicillin and 100 mg/mL streptomycin (Gibco, USA). HUVECs with a FRET sensor that allows for real-time detection of caspase-3 activation during apoptosis was used (HUVEC-C3) [173]. HUVEC-C3 cells were cultured in DMEM medium containing 500 mg/mL G-418 sulphate (Gibco, USA) to maintain the FRET sensor in the stable cell line. A suspension of cells at a density of approximately 1×10^7 cells per mL was injected into the microchannels, and the microfluidic chip was then incubated in the CO₂ incubator at 37 °C for 48 hours until an intact cell monolayer was formed. Only an intact monolayer of HUVECs was selected for performing the experiments.

The microfluidic experiments were conducted outside of the CO₂ incubator. DMEM with 10 mM HEPES (Sigma, USA) was used to maintain the pH of the culture medium at a physiological value of 7.3–7.4 under atmospheric conditions. A heating stage was used to maintain the temperature of the microfluidic chip and the medium reservoir at 37 °C for long-term circulation of culture medium in the microfluidic system.

2.4 Detection of endothelial cell apoptosis *via* the FRET sensor C3

In order to conduct a real-time detection of the HUVEC cells in the microfluidic chip, we have developed a HUVEC-C3 cell line transfected with the FRET biosensor C3. The biosensor C3 is a recombinant substrate protein for caspase-3 and consists of three parts: a donor cyan fluorescent protein (CFP), a 16 amino acid peptide linker containing the caspase-3 cleavage sequence Asp-Glu-Val-Asp (DEVD), and an acceptor yellow fluorescent protein (YFP). When HUVEC-C3 cells are alive, the CFP is excited by using a 433-nm excitation light, and its fluorescent emission energy is transferred to the YFP in the cells, resulting in a

green fluorescent emission with $\lambda_{em} = 526$ nm. When caspase-3 is activated as the cells undergoing apoptosis, the linker peptide between CFP and YFP is cleaved by the caspase-3, abolishing the FRET effect and resulting in a change of emission light from green to blue with $\lambda_{em} = 480$ nm.

To measure the FRET effect, the microfluidic chip with HUVEC-C3 cells was excited by using a fluorescence microscope with a band-pass filter (430 ± 10 nm). The emission images of YFP (530 ± 20 nm) and CFP (480 ± 15 nm) were recorded by a computer-controlled CCD camera (Diagnostic Instruments, Inc., USA). The digital fluorescence images were then processed by using the ImagePro Plus software (Universal Imaging Co., PA) to obtain the YFP/CFP emission ratio. To validate the FRET-sensor based apoptotic assay, the HUVEC-C3 cells were treated with 500 nM Taxol (Sigma, USA) or exposed to UV-light for 3 min as a positive control. For each chamber, 10 observation sites containing at least 200 cells were counted. Apoptotic ratio was normalized by determining the ratio between the number of CFP cells and the total number of cells.

2.5 Hoechst staining

Normal HUVEC cells on the petri dish were pre-treated with Hoechst 33342 ($0.5 \mu\text{g/mL}$) and incubated for 30 min. Cells were trypsinized, washed with observation medium and injected into the microchannel in the presence of observation medium. The Hoechst dye molecules can be well maintained by the endothelial cells grown in the microchannel and the fluorescence of these dye molecules will not be affected by the flow of the medium in the microchannel. I compared the fluorescence image of the Hoechst-stained nucleus with

the phase image of the endothelial cell (ECs), thus verifying that HUVEC-C3 cells were suitable for these experiments.

2.6 Apoptosis inhibition by antioxidants

An antioxidant is capable of inhibiting the oxidation of other molecules, which is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Antioxidants were used to test whether oxidative stress is the cause of endothelial cell death. The endothelial cells were pre-treated for 1 hour with different kinds of antioxidants, including 100 μ M butylated hydroxyanisole (BHA), 1 mM L-glutathione reduced (GSH), 20 μ M propyl gallate (PG) and 1 mM N-acetylcysteine (NAC), respectively. After the treatment, the cells were subjected to different combinations of pulsatile flow profiles and glucose concentrations. Finally, the apoptotic rates were measured and analysed.

2.7 Preparation of plasma from healthy volunteers and DM patients

Plasma samples from healthy volunteers and six DM patients were obtained from the diabetes and endocrine centre at Tan Tock Seng Hospital. The whole blood samples were immediately centrifuged at 4 °C. The supernatant plasma was collected in glass tubes and stored at -20 °C for long-term storage and transportation. The plasma obtained from healthy volunteers was applied as the control group and the plasma from DM patients as the experimental group. The human blood plasma was pre-warmed to 37 °C and mixed with serum free DMEM-HEPES to 10% (v/v). Additional glucose solutions were added to match the glucose level of the medium to the original glucose level of the blood sample.

2.8 Inhibition of apoptosis by a pan-caspase inhibitor

To prove that pulsatile shear stress and the plasma samples themselves can induce caspase-dependent apoptosis, the caspase inhibitor Z-VAD-FMK was used to determine whether it could reduce the rate of EC apoptosis. HUVEC-C3 cells grown inside of a microfluidic chip were pre-treated with 40 μ M of the pan-caspase inhibitor Z-VAD-FMK for 2 hours. Cells were then treated with culture medium containing 10% DM patient plasma and subjected to a pulsatile fluidic shear force treatment for 8 hours. Afterwards, the microfluidic chip was removed from the system and incubated for 40 hours in a heating chamber, which maintained a temperature of 37 °C and a similar humidity to that in a CO₂ incubator. Finally, apoptotic rate of HUVEC-C3 cells was determined by FRET imaging analysis.

2.9 Proteomics and Western blot analysis

Two-dimensional differential in-gel electrophoresis (2D-DIGE) was performed by Applied Biomix (Hayward, CA, USA) to identify protein biomarkers in the plasma of patients with type 2 diabetes. This study used two plasma samples from patients with glucose levels of 18.8 mM and 12.6 mM and one sample from a healthy volunteer. Proteins from each sample were separately labelled with Cy2 (excitation wavelength = 492 nm, emission wavelength = 510 nm), Cy3 (excitation wavelength = 550 nm, emission wavelength = 570 nm), and Cy5 (excitation wavelength = 650 nm, emission wavelength = 670 nm). The labelled proteins from 3 samples were mixed and run on a 2D-DIGE with isoelectric focusing as the first

dimension and SDS-PAGE as the second dimension. Fluorescence images were captured using a Typhoon image scanner and analysed using ImageQuant software. The ratio of protein expression between the patient samples and the control sample was quantified using DeCyder software analysis. Proteins that were up-regulated or down-regulated by 1.5-fold with $p \leq 0.05$ compared with the control samples were selected. The protein spots were cut out and subjected to in-gel trypsin digestion followed by protein identification using MALDI-TOF/TOF mass spectrometry in Applied Biomics (Hayward, CA, USA).

To validate the expression levels of biomarkers identified by 2D-DIGE in clinical samples, the plasma containing 100 µg of total protein were separated using 10% SDS-PAGE and transferred onto Amersham Hybond ECL nitrocellulose membranes (GE Healthcare, WI). After blocking the membranes with 5% non-fat milk, the membranes were incubated with primary antibodies from Abcam, UK against haptoglobin, clusterin, apolipoprotein C-III, and transferrin at 1:1000 (v/v) dilutions overnight at 4 °C. The membranes were then incubated with HRP-conjugated secondary antibodies at 1:5000 (v/v) dilutions at room temperature for 1 hour and developed using an ECLTM Western-blotting Analysis System (ThermoFisher Science).

2.10 Statistical analysis

All data are presented as the mean $\pm$ SD of at least three independent experiments. Statistical significance was analysed using a two-tailed Student's *t* test. Only data with $*p \leq 0.05$ and $**p \leq 0.01$ were considered to be significant compared with the control group.

Chapter 3

**Studying the effects of pulsatile shear stresses and
hyperglycemia on endothelial cell apoptosis**

3.1 Introduction

In Chapter 1, I have discussed the ROS generation and mitochondria fusion in endothelial cells under hyperglycemia and pulsatile shear stresses. The appropriate pulsatile shear stress is a critical factor in the development of blood vessel structure either during early growth or adulthood [174,175]. Furthermore, some researchers have found that exposure to high level shear stress caused oxidative stress on the endothelial cells and further induced cell apoptosis or necrosis [176], but those studies were performed under laminar shear stress with either constant or variable flow rates that are different from the real physiological conditions in the blood vessels, which experience pulsatile shear stress. Thus, it is necessary to introduce pulsatile shear stress into the model to reflect the fluctuating flow rate of the blood stream in a human body. Moreover, the combinational effect of the two factors: hyperglycemia and shear stress, which compose the microenvironment of endothelial cell growth on endothelial cells, is still not clear.

In this chapter, the previous hemodynamic lab-on-a-chip system was modified in which one microfluidic chip can achieve multiple flows at the same time. The effects of various pulsatile shear stress profiles either alone or in combination with different glucose concentrations on endothelial cells apoptosis were examined.

In order to detect apoptosis in single living cells, I have applied the fluorescence resonance energy transfer (FRET)-based biosensor that could detect caspase activation in HUVEC-C3 cells. With this FRET-based biosensor cell line, no pre- or post- staining is needed; meanwhile, a real-time observation of cell apoptosis can be achieved.

In this highly controllable microfluidic system, shear stress was directly imposed onto the endothelial cells' surface and a high concentration of glucose was added into culture medium to mimic the physical and chemical stresses applied to the cells. The combination of these diabetes-mimicking microenvironments may promote intracellular ROS generation, which can in turn overload the antioxidant defence system of the cells, leading to endothelial cell dysfunction through the caspase-3 activation pathway [177]. A novel biosensing assay based on caspase-3 activity, which can detect apoptosis-activating compounds in a high throughput manner with a genetically engineered cell line, was integrated into the platform. The platform can achieve a real-time *in vitro* and convenient method for cell dysfunction detection.

3.2 Results

3.2.1 Hemodynamic Lab-on-a-chip system

The hemodynamic microfluidic chip system is shown in Figure 3.1a. The microfluidic chip system consisted of four rows of microchannels; and each microchannel has three microchambers, which serve as the cell culture sites. Each microchamber has a width of 600 μm , a length of 3 cm and a height of 150 μm . The separated microchannels were connected by flexible connectors. The connection condition and sequence of the channels can be easily adjusted, which facilitates the conducting series operation of fluorescent dye staining and washing, and also allows experiments to be performed in parallel under different flow conditions [178].

A simple illustration of the experimental process by using a three-row microfluidic chip is shown in Figure 3.1b [178]. Firstly, endothelial cells were loaded into the microchip with all the three microchannels being connected in series. After the cells reached confluency, the culture rows were placed under different pulsatile flow conditions and the flow conditions of each row can be changed without affecting other rows. The flow condition was controlled by a pulsation free precision pump (Nemesys, Cetoni), with a flow profile mimicking the blood flow in the blood vessel. Three flow profiles can be experimented on the endothelial cells simultaneously with three rows of microchannels, e.g., (1) a 12 h pulsatile shear stress condition of 15 dyne/cm² (SS15) with a normal heart rate of 70 beats per minutes (bpm), which represents a human in resting condition; (2) a 1 hour pulsatile shear stress condition of 30 dyne/cm² (SS30) with a heart rate of 140 bpm, which represents a human in exhaustive exercise condition, and subsequently 11 hours of SS15; and (3) 2 hours of SS30 and subsequently 10 hours of SS15. Initially, row 1 was connected to the syringe, which exerted a shear stress profile of SS15. Rows 2 and 3 were interconnected to the syringe, which exerted a shear stress profile of SS30. After 1 hour, row 2 was disconnected from row 3 and connected to row 1 for SS15. Similarly, after 2 hours, row 3 was connected together with rows 1 and 2 for SS15. As a result, all three flow profiles can be easily realized in a single microfluidic chip system.

The number of microchannel rows can be increased when more complex and varying flow condition processes are needed. By using this microfluidic system, a single microfluidic chip can not only perform a series of experiments with different pulsatile flow conditions

simultaneously, but also realize the parallel comparison among several cell culture chambers within one experimental condition.

Figure 3.1 Design of microfluidic chip. Schematic illustration of (a) hemodynamic microfluidic chip system, and (b) experimental process to study endothelial cell death under different pulsatile shear stress conditions [178].

3.2.2 Measurement of cell apoptosis by caspase-3 activating sensor

In this study, caspase-3 activation was used as an indicator for cell apoptosis. Human umbilical vein endothelial cells producing the FRET-based caspase-3 sensor (HUVEC-C3) were used for detecting the caspase activation in live sensor cells under various conditions. Cells grown in the microchannel were treated with culture medium contain three concentrations of glucose (5, 10, 20 mM) for 48 hours. And the effects of different glucose concentrations and hemodynamic conditions in inducing cell apoptosis were determined by measuring the FRET effects of the sensor cells.

According to the principle of FRET-based caspase sensor, the healthy sensor cells should emit more green lights due to the energy transfer between CFP and YFP. When the caspase-3 is activated during apoptosis, it will cleave the linker between CFP and YFP to eliminate the FRET effect. As a result, the sensor cells should emit more blue lights. The FRET effect can be easily quantified at single cell by merging the fluorescent image of YFP and that of CFP. As shown in Figure 3.2, the control HUVEC-C3 cells appeared in green color and the cells changed into blue color when they die by 12 h UV irradiation-induced apoptosis (Figure 3.2).

Figure 3.2 The fluorescence of HUVEC-C3 cells changes from green to blue in response to cell apoptosis induced by 12 h of UV-irradiation.

The FRET changes in response to the caspase-3 activation with three different glucose concentrations under three different exhaustive pulsatile flow profiles were investigated. The statistical results are shown in Figure 3.3 and 3.4. Firstly, the percentage of apoptotic cells under different glucose concentrations (5, 10 and 20 mM) with an exhaustive flow profile of 4 hours, exhaustive pulsatile flow condition of shear stress 30 dyne/cm^2 , and 8 hours normal pulsatile flow condition of shear stress of 15 dyne/cm^2 , were compared and analyzed. The YFP/CFP emission ratio, which indicated the caspase-3 activation in living apoptotic cells, apparently decreases with the elevation of glucose concentration. To observe a more obvious FRET change, the final YFP/CFP emission ratio was detected 48 hours after the treatment to provide more time for caspase activation. By increasing the glucose concentration from the normal physiological level of 5 to 10 mM, a nearly 2-fold increase of the apoptotic rate was observed (from 7.2 to 14%) under shear stresses of 4 h-SS30 and 8 h-SS 15 (Figure 3.3). Under the highest glucose concentration of 20 mM, about 22.5% of the endothelial cells underwent a caspase-3 related apoptotic cell death, which could significantly affect the

maintenance of the normal blood vessel function. Secondly, the apoptotic rate of endothelial cells under the same glucose concentrations of 20 mM with different pulsatile flow profiles were compared and analysed. It can be seen that cell apoptosis via the caspase-3 pathway is minimal under a normal pulsatile condition of shear stress of 15 dyne/cm² for 12 hours. When a longer period of the exhaustive pulsatile shear stress of 30 dyne/cm² was applied to the cells in the presence of 20 mM glucose, the measured apoptotic rate was significantly increased from 4% to 12.5% (Figure 3.4). Adding 2 hours of exhaustive pulsatile shear stress nearly doubled the apoptotic rates of the endothelial cells in all three glucose concentrations (Figure 3.4). These results show the potentially damaging effect of excessive mechanical force to endothelial cells, especially in combination with high concentration of glucose at 20 mM. For diabetic patients with a high glucose concentration in the blood vessels, it is quite dangerous to have an exhaustive blood flow lasting a long time, which will induce a high rate of apoptosis of the endothelial cells. When the exhaustive blood flow time becomes longer, it is more dangerous to diabetes patients. Two hours exhaustive blood flow under high glucose concentration may induce quite serious damage to the blood vessels.

Figure 3.3 FRET changes in response to caspase-3 activation with different glucose concentrations 5 mM, 10 mM, and 20 mM under shear stress 30 dyne/cm² for 4 hours and shear stress 15 dyne/cm² for 8 hours [178].

Figure 3.4 FRET changes in response to caspase-3 activation with 20 mM glucose under different treatment conditions with pulsatile shear stress of 30 dyne/cm² and 15 dyne/cm² for 0+12 hours, 1+11 hours, 2+10 hours, and 4+8 hours [178].

3.2.3 Inhibition of the apoptotic effect *via* antioxidants

To determine the importance of the ROS production and the oxidative stress to the endothelial dysfunction, the effects of four antioxidants were examined, i.e., 100 μ M butylated hydroxyanisole (BHA), 1 mM L-glutathione reduced (GSH), 20 μ M propyl gallate (PG) and 1 mM N-acetylcysteine (NAC). In these experiments, the endothelial cells were pre-treated with each of the antioxidants for 1 hour and subsequently, were exposed to an exhaustive pulsatile shear stress of 30 dyne/cm² for 2 hours followed by a normal shear stress of 15 dyne/cm² for 10 hours (Figure 3.5) [178]. To evaluate the effect of these anti-oxidants, the YFP/CFP emission ratio was detected immediately after the treatment. The apoptotic rate was significantly reduced by PG but not the other three antioxidants. Our previous studied showed that PG can prevent Bax translocation to mitochondria and cytochrome c release, which are the two upstream events of caspase-3 dependent cell apoptosis [179].

Figure 3.5 Effect of antioxidant drugs: negative control, PG, GSH, BHA, and NAC on high glucose and shear stress induced cell apoptosis [178].

3.3 Conclusions and discussions

In this chapter, a novel endothelial cell apoptosis detection method, which combines a caspase-3-based FRET biosensor cell line and the microfluidic chip system, was developed. The apoptosis and necrosis of endothelial cells undergo a series of different pulsatile flow conditions and glucose concentrations can be detected with high accuracy since the FRET biosensor provides real-time observation and does not need post-treatment. The results show that high glucose concentration could significantly increase endothelial death, which was further increased by including the effect of the pulsatile shear stress. Using the endothelial cell line transfected with the FRET biosensor C3, a more accurate cell apoptotic percentage can be obtained with direct observation of fluorescence emission changes. The activation of the biosensor also confirms the endothelial cell apoptosis induced by the elevated pulsatile shear stress and high glucose concentration is through the caspase-3 pathway. This information can form the basis for determining how hyperglycemia as the major risk factor causes endothelial dysfunction under a physiological pulsatile blood flow condition, which leads to cardiovascular diseases and other complications associated with diabetes.

Chapter 4

Studying the effects of plasma from patients with diabetes on endothelial cell apoptosis

4.1 Introduction

As discussed in Chapter 1, the vascular complications are the main causes of significant morbidity and mortality in diabetes mellitus. Many studies have suggested that abnormal high blood glucose concentration (hyperglycemia) is the primary cause of the development and progression of the complications of DM originating from endothelial cell dysfunction. In Chapter 3, I have discussed that another physiological factor, the shear stress along the endothelium, is significant in the formation of blood vessel structures during early development and for the repair of damaged blood vessels. Endothelial cells can alter their morphology and function in response to shear stress and these responses are closely related to flow-regulated phenomena such as angiogenesis, vascular remodelling and atherosclerosis [138,175,180]. Furthermore, the pulsatile nature of blood flow plays a critical role in revealing the elaborate effects of fluid shear stress. In Chapter 3, I have introduced a novel hemodynamic lab-on-a-chip system to investigate how pulsatile shear stresses could influence endothelial cell functions. I have applied different fluid shear stresses and glucose concentrations to determine the ROS generation, mitochondrial dysfunction [125] and endothelial cell apoptosis [178] under hyperglycemic conditions. The additive effects of physical and chemical stresses on endothelial cell dysfunction have been observed.

Besides the pulsatile shear stresses and glucose levels presented in human's blood, other blood components also play indispensable roles in vascular function and in most metabolic diseases, including hypocalcaemia, hypoglycaemia, obesity, hyperlipidaemia and metabolic arthritis [181-185]. Hyperglycaemia is the major manifestation of diabetes. However, the vascular complications of diabetes are related to various proteins in the blood and

comorbidities inclusive of hyperlipidaemia and hypertension. How these proteins function under dynamic conditions still remains unknown.

In this chapter, I investigated the effect of blood proteins in inducing diabetes-associated endothelial cell apoptosis by applying the plasma from healthy volunteers and patients with diabetes in microfluidic system (Figure 4.1). Pulsatile shear stresses were introduced into the endothelial cell surface, and human plasma was mixed with culture medium to simulate the microenvironment of pathological blood including physical, chemical and biological factors. This investigation may help to identify the most prominent cause of EC death in the vascular microenvironment of patients with diabetes, to reveal the interactions among physiological factors in promoting EC apoptosis and to elucidate the mechanism of how blood components act under hemodynamic conditions.

Figure 4.1 Schematic illustration of the mechanisms by which blood components and shear stresses induced endothelial cell death [172].

4.2 Results

4.2.1 Construction of hemodynamic lab-on-a-chip system

The hemodynamic lab-on-a-chip system is shown in Figure 4.2A. The microfluidic chip was fabricated from polydimethylsiloxane (PDMS) material using standard soft lithography processes [186]. The PDMS slab was bonded to a poly-lysine coated glass slide by exposure to air plasma for 15 seconds using a handheld corona treater. The microfluidic chip consists of three rows of microchannels that enable triplicate experiments to be performed at the same time. Each row has three different microchannel designs that mimic different blood vessel shapes (Figure 4.2B and 4.2C), and these microchannels are sites for EC culture. Microchannel design 1 has a width of 600 μm , a height of 150 μm and a total length of 3 cm to mimic normal human arteries. Microchannel design 2 has a width of 600 μm at the

beginning of the channel and gradually becomes narrower, reaching 300 μm in the middle of the channel. In microchannel design 3, I introduced a half-circle block with a radius of 300 μm in the middle of the channel to mimic the blood vessel with an atherosclerotic plaque (Figure 4.2C). The height and the total length of designs 2 and 3 remain the same as design 1.

Figure 4.2 Design of the microfluidic system. (A) Schematic illustration of the hemodynamic system, (B) flow process and connection method in the experiments, allowing for a very small volume of liquid to circulate in the system, and (C) phase image of microchannel designs 1, 2 and 3 with observation sites circled [172].

According to Poiseuille's equation for calculating the shear stress of a channel with a rectangular shape [178], $\tau = 12Q\mu/h^2w$, where Q is the volume flow rate, μ is the viscosity

of culture medium, which is 1.2 MPa s in the experiments, h is the height of the microchannel at the fixed size of 150 μm , and w is the width of the microchannel. As all three channel designs have the same parameters except for the reduced width in some areas of designs 2 and 3, those channels feature greater shear stresses.

The experimental procedure using the multi-channel microfluidic chip is illustrated in Figure 4.2B. This optimised connection of the pulsation-free pump and microfluidic chip allows a very small amount of medium to circulate in the system and reduces the chance of contamination upon exposure to the environment. Before the introduction of the pulsatile flow, ECs were injected into all the microchannels to form a blood vessel-like monolayer. As shown in Figure 4.2B, the two syringes on the pulsation free pump (neMESYS, Cetoni) were connected with three-way valves. Syringe I was used to collect the medium from the medium reservoir. Syringe II was used to inject the medium directly into the microfluidic chip to generate pulsatile flow. Once the two syringes approached their capacities, the two three-way valves were set so as to alter the connection to simultaneously change the flow direction. Consequently, the roles of these two syringes were reversed. In this experimental set-up, the volume of medium circulating in the system and the direction of medium flowing over the endothelial cell monolayer remained the same.

In the following experiments, two different flow profiles were applied that simulate the actual pulsatile blood flow in the human circulation. One is a pulsatile flow at an average flow rate of 1.41 $\mu\text{L/s}$, which produces an average shear stress of 15 dyne/cm^2 in 600 μm -wide channels with a pulse rate of 70 bpm. This condition mimics the blood flow in the artery of a healthy human body [187,188]. The other profile is a pulsatile flow at an average

flow rate of 2.22 $\mu\text{L/s}$ with a pulse rate of 110 bpm to simulate the blood flow in patients with diabetes with an increased resting heart rate, resulting in a higher average shear stress of 23.6 dyne/cm² in 600 μm -wide channels [189]. The average flow rate was interpreted into the typical flow velocity curve of the artery and imported into neMESYS software. The duration of the flow rate curve was set at 860 ms for 70 bpm and 548 ms for 110 bpm, respectively. As apoptosis is a programmed death event, caspase activation is expected to occur after cells being exposed to the death inducer for certain times. In this experiment, the cells were exposed to the shear stress treatment for 8 hours. Afterwards the microfluidic chips were removed from the system and incubated for 40 hours in a heating chamber, which maintained a temperature of 37 °C and a similar humidity to that in a CO₂ incubator.

4.2.2 Preparation of plasma from healthy volunteers and DM patients

Plasma samples from three healthy volunteers and six DM patients were obtained from the diabetes and endocrine centre at Tan Tock Seng Hospital. An ethical approval document was obtained from Domain Specific Review Board of National Healthcare Group of Singapore (DSRB, Ref 2012/02218), and all patients signed an informed consent form. The whole blood samples were immediately centrifuged at 4 °C. The supernatant plasma was collected in glass tubes and stored at -20 °C for long-term storage and transportation. The plasma obtained from healthy volunteers was applied as the control group (glucose level considered to be 5 mM) and the plasma from DM patients as the experimental group. The human blood plasma was pre-warmed to 37 °C and mixed with serum free DMEM-HEPES to 10% (v/v). Additional glucose solutions were added to match the glucose level of the medium to the

original glucose level of the blood sample. The demographics, physical characteristics and concomitant conditions in healthy volunteers and DM patients are summarised in Tables 4.1 and 4.2 respectively [172].

Table 4.1 Demographics, physical characteristics and concomitant conditions in healthy volunteers. [172]

	Volunteer 1	Volunteer 2	Volunteer 3
Age	62	63	27
Sex	Female	Female	Female
Ethnicity	Chinese	Chinese	Indian
BMI	28.9	23	25.9
Smoking status	Non-smoker	Non-smoker	Non-smoker
Concomitant hypertension	Yes	No	No
Concomitant hyperlipidaemia	No	Yes	No
ACE-I	Yes	No	No
Aspirin	Yes	No	No
Statin	No	Yes	No

ACE-I: Angiotensin Converting Enzyme Inhibitor, eGFR: Estimated GFR using the MDRD formula, BMI: Basal metabolic index calculated as $\text{weight in kg}/(\text{height in m})^2$

Table 4.2 Demographics, physical characteristics and concomitant conditions in the type 2 DM patients. [172]

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age	39	50	53	61	58	65
Sex	Female	Male	Male	Male	Female	Male
Ethnicity	Chinese	Malay	Chinese	Chinese	Chinese	Chinese
BMI	28.1	29.3	28.2	27.9	30.4	24.9
Smoking Status	Non-smoker	Non-smoker	Non-smoker	Smoker	Non-smoker	Ex-smoker
Hypertension	Yes	Yes	Yes	Yes	Yes	Yes
Hyperlipidaemia	Yes	Yes	Yes	Yes	Yes	Yes
Aspirin	No	Yes	Yes	Yes	Yes	Yes
ACE-I	No	Yes	Yes	Yes	Yes	Yes
ARB	Yes	No	No	No	Yes	No
Metformin	Yes	Yes	Yes	Yes	Yes	Yes
Sulfonylurea	Yes	No	Yes	Yes	No	Yes
Insulin	Yes	Yes	No	No	Yes	Yes
Statin	Yes	Yes	Yes	Yes	Yes	Yes
Capillary blood glucose(mM)	10.4	12.4	12.6	16.3	18.8	15.8
HbA1c (%)	10.0	9.6	11.2	9.2	11.5	10.6
eGFR	>60	50	45	>60	>60	38
Nephropathy	No	Mild	Mild	No	No	Mild
Retinopathy	No	No	No	No	No	No

ACE-I: Angiotensin Converting Enzyme Inhibitor, ARB: Angiotensin receptor blockers.

eGFR: Estimated GFR using the MDRD formula.

4.2.3 Higher levels of both glucose and pulsatile shear stress can induce apoptosis in endothelial cells

As I previously showed that high glucose and high shear stress could induce EC apoptosis, in this study I examined this issue under conditions that are closer to the clinical situation.

Three microchannels with different shapes were designed to generate different flow forces

mimicking the blood flow-induced shear stresses in normal, narrowed and partially blocked human arteries (Figure 4.2C). Glucose was added to the culture medium to match the blood glucose concentration of each of the six clinical samples. Pulsatile flow was introduced for 8 hours. After incubating cells under static conditions for an additional 40 hours, the apoptotic rates of HUVEC-C3 cells were determined by calculating the percentage of apoptotic cells. As shown in Figure 4.3, apoptotic cells appeared blue, while live cells appeared green in the FRET images. For each condition, 1000–2000 cells were analysed, and the quantitative data in Figure 4.6A showed that a higher glucose concentration induced more EC apoptosis [172].

Elevating shear stress by reducing channel dimensions significantly increased the rate of apoptosis, especially in cells treated with high glucose concentrations of 15.8–18.8 mM (Figure 4.3). For example, for cells treated with 18.8 mM glucose, the percentage of apoptosis increased sharply from 16.7% to 24.9% and 40.4% for channels 1, 2, and 3, respectively. In contrast, for cells treated with 12.4 mM glucose, the rate of apoptosis only increased slightly from 9.8 to 11.6% (Figure 4.6A). In addition to using FRET imaging to indicate caspase-3 activation, cells were stained with the DNA dye Hoechst 33342 as an apoptotic indicator measuring chromatin condensation and DNA fragmentation. A similar level of apoptosis was detected using this method (Figure 4.4). These results suggest that increasing shear stress is much more harmful to ECs at a high glucose concentration than it is at a low glucose concentration.

More importantly, under static conditions, treating cells with 18.8 mM glucose medium for 48 h only induced 7.32% HUVEC-C3 cells to undergo apoptosis, which is 5.5-fold less

effective than the pulsatile flow achieved in microchannel design 3 (Figure 4.6A). This observation further demonstrates the critical role of pulsatile shear stress in driving hyperglycaemia-induced EC death. This result indicates the combined effects of the physical force of shear stress and the chemical factor of high glucose in causing additive damage to the endothelium lining the inner layer of blood vessels in DM patients.

Figure 4.3 Merged images of HUVEC-C3 cells with FRET effects under different glucose concentrations and different microchannel designs using medium with 10% FBS and defined glucose concentrations [172].

Figure 4.4 (A) Hoechst staining and phase images of HUVEC cells under different glucose concentrations and different microchannel designs using medium with 10% FBS and defined glucose solutions of which the concentrations were equivalent to the plasma samples, and (B) The percentage of apoptotic cells determined by analysing 1,000-2,000 cells by Hoechst staining under different glucose concentrations and different microchannel designs ($p < 0.05$)

compared to 5 mM glucose in samples of A or B in microchannel design 1 ^{*}, design 2 [#] and design 3 ^Δ, respectively) [172].

4.2.4 Plasma from DM patients induced more cells to undergo apoptosis compared to medium with the same glucose concentration

Hyperglycaemia has been considered as a hallmark of diabetes. However, in the microenvironment of blood vessels, blood components including proteins, lipids and antioxidant vitamins should serve as factors protecting or damaging the endothelium. From this perspective, I combined clinically obtained plasma with the microfluidic system to investigate how these biological factors function in an experimental set up with pulsatile shear stress and hyperglycaemia. The experimental conditions were identical to those used with glucose medium, with the exception that 10% plasma from either healthy volunteers or DM patients was replaced with FBS.

I measured the rates of EC apoptosis in three different channel designs when exposed to the plasma of six DM patients or three healthy volunteers. Figure 4.5 presents representative FRET images from three different microchannel designs at four glucose concentrations. Figure 4.6B presents the results of quantitative analysis of 1000–2000 cells under each experimental condition. Although I observed that a higher glucose concentration significantly increased the rate of apoptosis as observed in the glucose medium experiment shown in Figure 4.6A, much higher levels of apoptosis were detected in cells treated with the high glucose patient's plasma compared with cells treated with glucose medium under the lower shear stress situation generated in channel designs 1 and 2 (Figure 4.6A and B). In these cases, the rate of apoptosis was 1.4–2.3 times higher for the patient's plasma compared

with the FBS medium containing the same concentration of glucose (Table 4.3). The additive apoptotic effect was not observed either for cells seeded in microchannel design 3, in which the higher shear stress already induced a high level of apoptosis, or for cells in the static condition (Figure 4.6 and Table 4.3).

The results obtained from human plasma suggest that other components of patients' plasma besides glucose can further damage ECs. These components can even carry out their harmful effects on ECs in microchannel designs 1 and 2, representing normal and the gradually narrowed blood vessels present in most patients with diabetes. As EC dysfunction can lead to many diabetes-related vascular diseases, the identities of these components should be investigated.

Table 4.3 Ratio of rate of apoptosis between DM patients' plasma and medium with FBS.

[172]

Glucose concentration	Microchannel design	Apoptotic rate – DM plasma	Apoptotic rate – FBS medium	Ratio of DM plasma/FBS
15.8 mM	1	23.3	11.5	2.0
	2	24.6	15.7	1.6
	3	23.0	23.9	1.0
16.3 mM	1	28.7	12.5	2.3
	2	29.0	20.3	1.4
	3	32.0	31.9	1.0
18.8 mM	1	30.1	16.7	1.8
	2	36.4	24.9	1.5
	3	43.1	40.4	1.1

Figure 4.5 Merged images of HUVEC-C3 cells with FRET effects in different microchannel designs using medium with 10% plasma from healthy volunteers or DM patients at defined glucose concentrations [172].

Figure 4.6 The percentage of apoptotic cells was determined by analysing 1,000-2,000 cells in the merged FRET images for each condition. (A) Medium with 10% FBS and glucose concentrations equivalent to the clinical samples, and (B) 10% plasma of healthy volunteers and type 2 DM patients ($p < 0.05$ compared to the first group in all samples of A or B in microchannel design 1 *, design 2 [#] and design 3 ^Δ, respectively) [172].

4.2.5 A sudden increase in shear stress induces more apoptosis in endothelial cells, which can be reduced by a caspase inhibitor

Before finding the pro-apoptotic factors in the plasma samples, I investigated the impact of sudden changes in the shear stresses applied to ECs. The shear stresses at the observation

sites in microchannel designs 2 and 3 were the same but induced different rates of EC apoptosis. This finding indicates that in the human body, pathological plaques possess a higher risk of causing endothelial destruction and ulceration. To further investigate the impact of this sudden change, the rates of apoptosis of HUVEC-C3 cells were compared between the observation site and its upstream or downstream site within the same microchannel (Figure 4.7A). The experiment was conducted using the same conditions described above using the DM patient's sample with 18.8 mM glucose. Merged images of FRET effects are shown in Figure 4.7B.

The bar graph in Figure 4.7C shows that 43.1% of HUVEC-C3 cells underwent apoptosis in the observation site at the point where the channel diameter suddenly decreases from 600 to 300 μm . A much lower rate of apoptosis of approximately 29% was found in areas either upstream or downstream of the partially blocked site, similar to the rate of 30.1% observed using the plasma sample with 18.8 mM glucose in microchannel design 1 (Figure 4.6B). This finding suggests that the sudden change in shear stress can damage the ECs directly exposed to these flow forces, but will not affect the ECs in the surrounding areas.

The pan-caspase inhibitor Z-VAD-FMK was used to determine whether this multi-factor-induced apoptosis could be reduced by inhibiting caspase-3 activation. HUVEC-C3 cells were pre-treated with 40 μM of the pan-caspase inhibitor Z-VAD-FMK for 2 hours and exposed to the flow of a plasma sample with 18.8 mM glucose. This result shows that the percentage of EC apoptosis was reduced from 43.1% to 24.1% (Figure 4.7D). This result confirms that EC apoptosis can occur under pulsatile shear stresses and the biochemical conditions present in the clinical plasma samples. Furthermore, it also suggests that

preventing caspase activation could be a therapeutic strategy for treating diabetic vascular complications by reducing EC apoptosis.

Figure 4.7 (A) Illustration of the observation site in microchannel design 3, (B) Merged images of HUVEC-C3 cells with FRET effects of different sites of microchannel design 3 in the presence of caspase inhibitor in the DM patient's plasma with 18.8 mM glucose, (C) The percentage of apoptotic cells determined by analysing 1,000-2,000 cells from the merged FRET images at each site ($*p < 0.05$ compared to the site before or after blocking along the flow direction), and (D) The percentage of apoptotic cells determined by analysing 1,000-2,000 cells in the merged FRET images with caspase inhibitor treatment ($*p < 0.05$ compared to the observation site) [172].

4.2.6 Identification of proteins with an altered expression profile in the plasma of DM patients

Little difference in cell death was observed between the glucose medium and patients' plasma in microchannel design 3. One possible reason for this is that the mechanical forces of the high flow rate reduced the interaction between plasma proteins and the endothelium. In this situation, the reduction in the interactions of “bad” proteins and thus their subsequent negative effects was greater than that of “good” proteins, resulting in a smaller increase in the rate of apoptosis of ECs.

To further investigate the biological factors of human plasma that can contribute to EC apoptosis under pathological conditions, I identified these plasma components by comparing the protein profiles between different samples. Proteins in the control plasma from a healthy volunteer were conjugated with Cy2 dye, which emits green fluorescence; proteins in the DM patient plasma with a glucose concentration of 12.6 mM were conjugated with Cy3 dye, which emits red fluorescence; and proteins of the DM patient plasma with a glucose concentration of 18.8 mM were conjugated with Cy5 dye, which emits far-red-fluorescence. Three groups of samples were prepared by mixing equal amounts of proteins from each plasma sample: (1) control vs. patient 3 with 12.6 mM glucose, (2) control vs. patient 5 with 18.8 mM glucose, and (3) patient 3 with 12.6 mM glucose vs. patient 5 with 18.8 mM glucose. The proteins in each group of samples were separated by 2D-DIGE based on their electrical charges and molecular weights. Fluorescence images of 2D-DIGE from the first two combinations of three dyes were merged to produce the images in Figure 4.8A.

In these merged images, red colour indicates up-regulated proteins, green represents down-regulated proteins, and yellow indicates equivalent expression between the patient and control samples. A total of 76 protein spots could be visualised in the merged images. The fluorescence intensity of each protein spot was quantified by DeCyder analysis. Out of 76 protein spots, the expression levels of 18 were consistently up-regulated or down-regulated at least 1.5-fold with $p \leq 0.05$ among all three groups: (1) 18.8 mM glucose/control, (2) 12.6 mM glucose/control, and (3) 18.8 mM glucose/12.6 mM glucose. These proteins were cut out and subjected to in-gel trypsin digestion followed by protein identification using MALDI-TOF/TOF mass spectrometry by Applied Biomics (Hayward, CA, USA).

Among the 18 identified protein spots listed in Table 4.4, 12 were differentially down-regulated and the other 6 were up-regulated. Among the 12 down-regulated protein spots, several were found to be the same protein that was separated by 2D-DIGE due to its different pI values. Specifically, proteins at spots 62–64 were all identified to be haptoglobin α_2 , proteins at spots 32 and 33 were both identified as haptoglobin β , and proteins at spots 37 and 38 were found to be clusterin.

Table 4.4 Information about proteins identified by 2D-DIGE.

Spot ID	Protein name	Ratio of protein levels			Biological function
		18.8/C	12.6/C	18.8/12.6	
29	Alpha-1-acid glycoprotein 2	-28.4	-4.5	-6.1	Carrier of charged lipophilic compounds
57	Apolipoprotein A-I	-5.6	-2.2	-2.4	High-density lipoprotein
43	Apolipoprotein E	-4.4	-2.3	-1.9	High-density lipoprotein
37	Clusterin	-15.4	-8.3	-1.8	Inhibiting apoptosis
38	Clusterin	-3.4	-1.9	-1.7	
32	Haptoglobin β	-22.4	-13.4	-1.6	Binding to free haemoglobin to inhibit oxidative stress
33	Haptoglobin β	-9.8	-6.0	-1.6	
62	Haptoglobin $\alpha 2$	-10.2	-4.0	-2.4	
63	Haptoglobin $\alpha 2$	-5.2	-2.2	-2.2	
64	Haptoglobin $\alpha 2$	-4.0	-1.5	-2.5	
44	Histidine-rich glycoprotein	-4.0	-1.8	-2.1	Modulating immune and vascular system
61	Retinol-binding protein 4	-6.5	-4.2	-1.5	Delivering retinol to liver
30	Alpha-1-acid glycoprotein 2	4.3	3.1	1.5	Carrier of charged lipophilic compounds
74	Apolipoprotein C-II	5.4	2.8	2.0	Very-low-density lipoprotein
72	Apolipoprotein C-III	4.4	1.6	2.9	Very-low-density lipoprotein
28	Beta-2-glycoprotein	2.6	1.6	1.7	Inhibiting coagulation factors
70	Haemoglobin β subunit	8.1	4.6	1.8	Inducing oxidative stress
4	Serum albumin	2.1	1.5	1.5	Transporting hormones, fatty acids

Western blot analysis was performed to validate the expression profiles of three representative proteins determined by 2D-DIGE, and positive confirmation was obtained for all three cases (Figure 4.8B and C). Both methods revealed that the levels of haptoglobin β and clusterin were reduced more significantly in the patient plasma with 18.8 mM glucose than in plasma with an intermediate level of glucose at 12.6 mM. In contrast, the level of apolipoprotein (Apo) C-III increased more prominently in patient samples with higher glucose.

Figure 4.8 (A) Fluorescence images of 2D-DIGE with over-expressed proteins in DM patients' plasma compared to healthy volunteer's plasma in red and under expressed proteins in green, (B) Western Blot analysis of the expression level of haptoglobin, clusterin and apolipoprotein C-III in three of the plasma samples, and (C) Histogram of normalised protein levels of the three plasma samples (* $p < 0.05$ and ** $p < 0.01$ compared to the group using the DM patient's plasma with a glucose concentration of 12.6 mM) [172].

After examining the functions of the proteins identified here, I realised that all eight proteins with beneficial effects on EC or vascular function were down-regulated, while another six proteins with harmful potential were up-regulated in the plasma of DM patients with hyperglycaemia. For example, the levels of two subunits of haptoglobin (β and $\alpha 2$),

with the function of binding and removing haemoglobin from the blood to reduce its oxidative stress were significantly decreased in DM patient's plasma with 18.8 mM glucose, while the level of “bad” haemoglobin β was considerably higher in the DM patient's plasma with higher glucose.

Other examples are the “good” proteins including clusterin, which can promote cell–cell adhesion and inhibit apoptosis by interacting with activated Bax [190], and the high density lipoproteins of Apo A-I and E, which can remove cholesterol from the blood, were all reduced in hyperglycaemia plasma. The “bad” proteins were present at higher levels in the DM patient's plasma with 18.8 mM glucose, including the low-density lipoproteins Apo C-II and C-III, which can promote the formation of atherosclerotic plaques.

Apolipoprotein C-III is a major component of the family of triglyceride rich lipoproteins. Apo C-III is elevated in metabolic syndrome and DM type 2 and correlates with BMI (Body Mass Index) and insulin sensitivity [191]. The concentration of Apo B with respect to that of Apo C-III is the strongest lipoprotein predictor of the progression of coronary atherosclerosis, even in patients where LDL-cholesterol (LDL-C) was aggressively lowered with lovastatin [192]. Apo C-III in high density lipoprotein-cholesterol (HDL-C) is also associated with coronary heart disease and exhibits a positive correlation with very low density lipoprotein (VLDL) and triglycerides. This suggests that Apo C-III exerts atherogenic properties beyond its effect on Apo B lipoprotein metabolism [193]. Apo C-III alone and Apo C-III rich VLDL increase monocyte adhesion to vascular endothelial cells and activate NF- κ B, thus leading to endothelial activation. Apo C-III HDL does not reduce

the adhesion of monocytes to vascular endothelial cells, whereas HDL-C with no Apo C-III reduces monocyte adherence, thus counteracting the benefit of HDL-C [194].

Haptoglobin is known to provide protection against haemoglobin peroxidation-triggered endothelial damage as both the intrinsic haemoglobin oxidation pathway (which generates toxic reaction products causing EC apoptosis) and extrinsic oxidation pathway (which generates lipid oxidation products) can be prevented in the presence of haptoglobin. The lack of haptoglobin is also known to be associated with unbalanced VEGF- α /angiopoietin-1 expression, intramural haemorrhage and impaired wound healing after myocardial infarction. Haptoglobin genotypes are also known to be associated with endothelial dysfunction and vascular complications, with haptoglobin genotype 2-2 associated with higher oxidative risk.

Clusterin has also been implicated in lipid metabolism and atherogenesis and is known to be protective. In fact, laminar shear stress is known to up-regulate clusterin, the complement-inhibitory protein, thus preventing endothelial activation through the complement cascade. In a study conducted in hypertensive Japanese women, polymorphisms in the clusterin gene influenced lipid levels and the thickness of the carotid intima media.

In one study, proteomic analyses of HDL-C from patients with coronary artery disease were compared to those of healthy individuals. HDL proteomic analyses and subsequent validation and functional characterisation suggested a reduced clusterin and increased Apo C-III content of HDL-C in patients with coronary artery disease when compared to normal individuals as mechanisms leading to altered effects on endothelial apoptosis.

In summary, this study established a positive correlation among protein expression profiles, glucose level, and apoptosis induction. The plasma of the DM patient with 18.8 mM glucose had an elevated level of proteins with negative functions (1.5–2.9-fold increase) and a reduced level of proteins with positive functions (1.5 to 6.1-fold reduction) compared with the plasma with 12.6 mM glucose (Table 4.4). The plasma with 18.8 mM glucose also induced a much higher level of apoptosis (30.1% to 43.1% for channel designs 1–3) than the plasma with 12.6 mM glucose (12.2% to 16.4% for channel design 1–3) (Fig. 4.6B).

4.3 Conclusions and discussions

In this chapter, I have investigated the elaborate effects of the pathological microenvironment inside the bloodstream of DM patients upon the induction of EC apoptosis. A hemodynamic lab-on-a-chip system was used to generate fluid shear stress simulating the pulsatile flow of the bloodstream at different flow and pulse rates. The sizes and shapes of microchannels were engineered to mimic the normal, narrowed or partially blocked blood vessels representing healthy or damaged blood vessels with increased shear stress. Plasma with various concentrations of blood glucose was obtained from DM patients and healthy volunteers and used to investigate the apoptotic effects of glucose and plasma components on EC. The results showed that more ECs underwent apoptosis if they encountered higher levels of physical factor of pulsatile shear stress or higher levels of chemical factor of glucose. The most important finding of this study is that in addition to hyperglycaemia, other plasma components can also contribute to additional EC apoptosis. By analysing the expression profiles of plasma samples, I identified fourteen proteins

including apolipoprotein C-III, haemoglobin β , haptoglobin, and clusterin, and the expression levels of these proteins were significantly altered in agreement with the concentration of blood glucose and the ability to stimulate apoptosis. If the expression profiles of these proteins can be validated using large numbers of patient samples, then the plasma levels of those proteins may serve as new indicators predicting the level of endothelial dysfunction in the arteries of DM patients, thus enabling clinical evaluation of the risk of vascular complications.

Chapter 5

Investigating the relationships between haptoglobin levels and the risk factors of diabetes

5.1 Introduction

From the results presented in Chapter 4, I can conclude that the blood components, especially the plasma proteins, are playing significant roles in inducing endothelial cell apoptosis, of which the majority belongs to the lipoprotein family. However, haptoglobin (Hp), is a plasma protein that binds extracorporeal hemoglobin (Hb) in a tight non-covalent complex (Hb-Hp). The amount of haptoglobin in human plasma ranges from 0.2 mg/ml to 1.4 mg/ml. The primary function of Hp is to prevent loss of free hemoglobin into the kidney. This conserves the valuable iron present in hemoglobin, which would otherwise be lost from the body. Once hemolysis happens, free hemoglobin passes through the glomerulus of the kidney, enters the tubules, and tends to precipitate therein (as can happen after a massive incompatible blood transfusion, when the capacity of haptoglobin to bind hemoglobin is grossly exceeded).

However, the Hb-Hp complex is too large to pass through the glomerulus. Thus Hp helps to conserve iron. Concentration of Hp rises in inflammatory conditions and decreases hemolytic anemia. The half-life of the Hb-Hp complex is about 90 min, the complex being rapidly removed from plasma by hepatocytes. Free Hp level or Hp binding capacity depicts the degree of intravascular hemolysis.

5.2 Results

5.2.1 Determine haptoglobin levels in patients with diabetes

Human plasma samples were collected from 10 healthy people and 17 patients with diabetes at TTSH. Western analysis was used to determine the haptoglobin β levels in those plasma samples (Figure 5.1). Average Hp level of healthy people was used as a control. All the results were normalized by this control.

Figure 5.1 Haptoglobin expression level of healthy volunteers and patients with diabetes. M represents the mixture of sample from healthy volunteers, which acts as a control.

I hypothesized that patients with longer history of diabetes may have a lower level of haptoglobin, as oxidative stress in the blood may damage red blood cells, after which free hemoglobin could be released. More molecules of Hp proteins will be used to bind to free Hb and consequently free Hp level will be decreased.

A reverse correlation was found between haptoglobin levels and diabetic durations in patients (Figure 5.2). Five out of 17 patients with longer than 10 year history of diabetes

exhibited more than 50% decrease in their haptoglobin levels compared to the control. As haptoglobin is an acute phase protein, this result indicates that long-term diabetes may have higher risk of hepatocyte injury or hemolytic anemia.

Figure 5.2 Correlations between haptoglobin levels and duration of diabetes.

I also checked whether there is a correlation between the level of glucose and haptoglobin in patients with diabetes, as high blood glucose level may produce extra oxidative stress. However, no linear correlation was found between haptoglobin levels and blood glucose levels in DM patients (Figure 5.3). Although there is no direct molecular pathway linking the haptoglobin and glucose metabolism, hyperglycemia can reduce the functions of hepatocytes.

Figure 5.3 Correlations between haptoglobin levels and blood glucose levels.

Aged people may have a lower level of haptoglobin as the productivity of Hp by hepatocytes can decrease. However, other tissues (lung, kidney) may help maintaining the Hp level. No clear correlation was seen between haptoglobin levels and age of patients (Figure 5.4). However, five out of 14 DM patients at the age > 55 showed more than 50% reduction of Hp level, which may suggest that higher risk of cardiovascular diseases exists in patients with diabetes who are elder than 55.

Figure 5.4 Correlations between haptoglobin levels and age of the patients.

Figure 5.5 Correlations between haptoglobin levels and gender of the patients.

There are reports indicating that male DM patients are more likely to have vascular complications than female diabetic patients. However, no clear correlation was observed between these two gender groups (Figure 5.5).

There is no report indicating the total number or potential risk of diabetes in ethnicity of Chinese, Malaysian and Indian. Total numbers of Chinese patients and Indian patients are top in the world, 98 million and 65 million respectively in 2013 [195,196].

Figure 5.6 Correlations between haptoglobin levels and duration of diabetes.

I found that the average Hp is similar between Chinese DM patients and Indian DM patients in this cohort (Figure 5.6). This kind of comparative study shall have more participants to show whether there is any critical differences between Hp level and ethnicity of DM patients.

Body mass index (BMI) is a measure of relative body weight based on an individual's mass and height. BMI is used to measure or predict obesity and obesity induced complications. There is no previous report indicating the relationship between Hp level and BMI.

Figure 5.7 Correlations between haptoglobin levels and patients' BMI.

There is no linear correlation between Hp level and BMI of patients in this study. However, most of DM patients have a BMI over 25 (overweight), which may indicate that obesity is closely related to diabetes (Figure 5.7).

Smoking is now proven to be an independent risk factor for diabetes, and amongst diabetics it increases the risk of complications. Both high levels of glucose in the blood and smoking damage the walls of the arteries in such a way that fatty deposits can build up much easier. Smoking is also proven to be a risk factor for insulin resistance. Patients who are insulin resistant cannot use their bodily insulin properly.

Figure 5.8 Correlations between haptoglobin levels and smoking status of the patients.

Average level of Hp in non-smoker is higher than ex-smoker (Figure 5.8), indicating that smoking may also influence the level of blood components. This investigation may also need a larger amount of individuals to see if there is any critical difference between Hp level and ethnicity of patients.

Hemoglobin A1c (HbA1c) is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. As the average amount of plasma glucose increases, the fraction of glycated hemoglobin increases in a predictable way. This serves as an indicator for average blood glucose levels over the previous months prior to the measurement.

Figure 5.9 Correlations between haptoglobin levels and HbA1c% of patients.

There is no clear correlation between Hp level and glycated Hb level (Figure 5.9). There is an article reporting that Hp level is related to HbA1c levels but not the glycated albumin level [197]. However, this study was conducted in the same Hp phenotype.

5.2.2 Higher levels of both glucose and pulsatile shear stress can induce apoptosis in endothelial cells

In the previous studies, I showed that both high glucose and plasma proteins from DM patients can induce endothelial cell apoptosis. In order to study the apoptotic effects of plasma protein on ECs, we collaborated with Professor Lindsay Brown whose group has established an obese rat model. They showed that chronic feeding of rats with a high-

carbohydrate, high-fat diet induces metabolic syndrome, including abdominal obesity, hypertension, glucose intolerance/insulin resistance and fatty liver, with relatively small increases in plasma glucose concentrations [198]. In addition, they have shown that components of food, such as anthocyanins from purple carrots [199], rutin and quercetin from onions [200,201], ellagic acid from strawberries [202] and omega-3 fatty acids from chia seeds [203] can reverse some or all of these symptoms of the metabolic syndrome in these rats. All compounds reduced inflammatory cell infiltration into the heart, liver and fat pads, and, when tested, all compounds reduced expression of the master-switches for both inflammation (NF κ B) and oxidative stress (Nrf2, nuclear factor-erythroid 2-related factor 2) [201,202].

In their studies, the male Wistar rats (8–9 weeks old) were randomly divided into the following 4 groups (n=12 for each group):

- 1) Control containing regular rat chow and serve as control rats.
- 2) H-diet containing high-carbohydrate, high-fat diet including condensed milk (39.5%), beef tallow (20%), and fructose (17.5%) together with 25% fructose in drinking water for 16 weeks and serve as diabetic rats.
- 3) H-diet+intervention containing H-diet for 8 weeks and feed with H-diet food with an intervention (for example 0.8 g/kg quercetin or 0.8 g/kg quercetin for another 8 weeks).

All the rats were monitored daily for body weight, food, and water intake. At 16 weeks, rats were deprived of food and sugar for 12 hours. Rats were terminated by euthanasia, and ~6 mL of blood samples were collected in a heparinized tube. The plasma were obtained after centrifugation at 5,000 rpm for 15 min and sent us for further analysis. I have diluted

the rat plasma to 10% in DMEM medium and applied it to our FRET sensor-based hemodynamic microfluidic system (Figure 5.10). The results showed that the apoptotic rates were 2.9% with control rat, 11% in diabetic rat, and 4.4% in diabetic rat feed with antioxidant agent, quercetin (Figure 5.12A).

Figure 5.10 Phase and FRET-merged images of endothelial cells treated with the plasma of rats from control group, HCHF group, and HQ group under the exposure of shear stresses at 15 dyne/cm² for 8 hours.

The Haptoglobin expression level in all rat plasma samples have also been determined by Western blot (Figure 5.13). Results showed that in HCHF rats, average Hp level is lower than control group. However, in HQ group Hp expression level returned back or even higher than control group, which indicated that the application of antioxidant may assist in adjusting Hp levels in individuals and improving the redox status of plasma.

Figure 5.11 Haptoglobin expression level samples of rat plasma by Western blot analysis.

Figure 5.12 A) Apoptotic rate of medium with rat plasma on endothelial cell apoptosis under pulsatile shear stresses, and B) Haptoglobin β expression level of rat plasma by Western blot analysis. * $p < 0.05$.

Figure 5.13 A) Haptoglobin β expression level of all rat plasma by Western blot, and B) Statistic analysis of Haptoglobin β expression level. * $p < 0.05$.

5.3 Conclusions and discussions

In this chapter, I have investigated the haptoglobin level in a group of human patients as well as in rats treated with different diets. Investigations on haptoglobin level of human patients with diabetes revealed that haptoglobin level may relate to the diabetes duration of patients. Patients with longer duration of diabetes mellitus may have a lower level of haptoglobin, suggesting a higher risk of oxidative stresses in the body. Meanwhile the verification of correlations between haptoglobin level and other demographic characteristics should rely on the experiments performing on the larger amount of patients.

Investigations in haptoglobin level of rats treated with different diets indicated that high-carbohydrate-high-fat diet can reduce the expression level of Hp in rats and increase the oxidative stresses in individuals, while antioxidant treatment can reverse this effect, thus reducing the oxidative stresses.

Type 2 diabetes is a complicated metabolic disease. I expect that the diet-induced diabetic rat can be used as animal models for a pilot study in investigating the effect of high-carbohydrate-high-fat diet on endothelial cells function. Whether the intervention with nutritional antioxidants can bring the concentrations of multiple demographic characteristics back to the normal ranges will be very valuable for developing specific therapies to prevent/treat vascular damage, which can reduce or slow down the development of diabetes-related complications including blindness, kidney failure, cardiovascular diseases, etc. The combination of the hemodynamic microfluidic system and diabetic animal model will provide an effective platform for screening nutritional antioxidants for diabetes therapy.

Chapter 6

Conclusions and future works

As a chronic metabolic disease causing more and more mortality, diabetes has now severely affected human's health. Although there has been greater emphasis by publics and scientists, neither the total number of patients have declined nor has the disease been kept under control. Over these several decades, the rapid development of biomedical science and technology has provided researchers many opportunities to investigate diabetes and its complications. However, from current common views and strategies, the therapeutic methodology towards diabetes is still traditional and conservative. Clinicians will prescribe diabetic patients with insulin injections, while adjuvantly taking metformin or other oral drugs. Interventions and improvements in lifestyles will also contribute to the treatments for diabetes. The ultimate purpose of these methods is to supply insulin or to minimize the risk of dysfunction of insulin uptake. Probably, the most effective methods to completely cure diabetes and its complications will be achieved by recovering the functions of pancreas beta cells and declining the oxidative stresses in human body. These are also the directions in biomedical research where numerous endocrinologists and scientists in metabolic diseases are steering towards.

In this dissertation, I presented a methodology which combines engineering and biomedical science, which is to use microfluidics to mimic blood microenvironments of the human body. I started my research from investigating the effect of high glucose levels on endothelial cells, assisted by dynamic flows which precisely mimicked the pulsatile nature of blood flow. With this innovation, I applied many plasma samples from patients with diabetes to further elucidate the combinational effects of physical, chemical and biological factors in the plasma by microfluidics and molecular biology experiments. I have proved

that hyperglycemia and abnormal pulsatile shear stresses can induce ROS generation, mitochondria fission, and apoptosis in endothelial cells. Comparative experiments using FBS and human plasma further demonstrated that abnormal levels of plasma proteins are significant in causing the dysfunction of endothelial cells, of which the lipoprotein family and haptoglobin are the most prominent of all and have attracted more attention from endocrinologist and researchers in metabolic diseases.

Our results have also provided evidence about the significant roles of antioxidants in preventing endothelial cell dysfunctions. Oxidative stresses are closely related to aging, obesity, and vascular complications of diabetes, but many still do not recognize the importance of taking antioxidants to meliorate the blood microenvironment. I am contented to see some researchers beginning to conduct clinical trials on patients with diabetes using Vitamin C, Vitamin D, and other antioxidants recently [204,205]. Furthermore, some small antioxidative molecules enriched in functional food like quercetin, and even the application of antioxidative plasma protein haptoglobin, should be further investigated. Convincingly, they are of promising values in both markets and clinical research.

Meanwhile, diabetic rats are excellent models in studying and validating the experimental results obtained from microfluidics. The optimized diabetic rat models can be applied in investigating the effect of high-carbohydrate-high-fat diet on endothelial cell function as pilot studies. As the glucose levels in rats cannot reach as high as humans, these results could serve as reliable controls that eliminate the interference of hyperglycemia. Based on these data, researchers can then further investigate whether adding different blood

components can induce / inhibit endothelial cell apoptosis, thus inducing / inhibiting the vascular complications of diabetes.

Last but not least, I also hope this dissertation can provide proof-of-concept evidence for the potential applications of microfluidic tools in more clinical research, including but not limited to endothelial cell functions, angiogenesis and blood rheology. Miniaturization will have an immeasurably broad market prospects in the rising directions of biomedical science and technology.

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List of Publications

JOURNAL ARTICLES

1. **Liu XF**, Chiew GYG, Fu AF, Han WT, Kamm RD, Luo KQ (2015) Study of liver tumor-induced endothelial tube formation in microfluidic system. (In preparation)
2. **Liu XF**, Yu JQ, Dalan R, Liu AQ, Luo KQ (2014) Biological factors in plasma from diabetes mellitus patients enhance hyperglycaemia and pulsatile shear stress-induced endothelial cell apoptosis. *Integrative Biology*. 6(5): 511-522. IF: 3.756.

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3. Yu JQ, **Liu XF**, Chin LK, Liu AQ, Luo KQ (2013) Study of endothelial cell apoptosis using fluorescence resonance energy transfer (FRET) biosensor cell line with hemodynamic microfluidic chip system. *Lab on a Chip* 13(14): 2693-2700. IF: 6.115.

CONFERENCE PAPERS AND PRESENTATIONS

4. **Liu XF**, Fu AF, How YH, Luo KQ (2014) Effect of hemodynamic shear stresses on circulating tumor cells. MicroTAS 2014, October 26-30, 2014, San Antonio, USA.
5. **Liu XF**, Fu AF, Luo KQ (2013) Application of a FRET-based biosensor in studying shear stress-induced apoptosis in circulating tumor cells. The 15th International Conference on Biomedical Engineering (ICBME 2013), December 4-7, 2013, Singapore.
6. Luo KQ, **Liu XF**, Yu JQ, and Dalan R (2013) The study of endothelial cells dysfunction in diabetes mellitus on chip. The 14th International Conference on Biomedical Engineering (ICBME 2012), January 10-12, 2013, Hong Kong.

7. **Liu XF**, Yu JQ, Dalan, R, Luo KQ (2012) Study the effects of blood samples from patients with diabetes on endothelial cell apoptosis using a hemodynamic Lab-on-a-chip system, The 2nd International Conference on Optofluidics, September 12-14, 2012, Suzhou, China.