CHAPTER V

DISCUSSION

In the present study, the experiments were conducted to investigate the effects of vitamin C supplementation on relationship of leukocyte adhesion to iris blood-flow perfusion in diabetic rats. From the results, it could be discussed as following:

I. The metabolic changes in diabetic model : Role of vitamin C

Streptozocin (STZ) had highly specific cytotoxic action on the Islets of Langerhans. This drug has been widely used to experimentally imitate Type I diabetes mellitus (IDDM) in rats. Numerous studies are performed with this model, providing its usefulness in studying diabetic complications. STZ action on β-cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Finally, hyperglycemia develops and blood insulin level decreased (West E et al., 1996). These changes in blood glucose and insulin concentration reflect abnormalities in β-cell function. In the present study, hyperglycemia occurs within 48 hours after the single dose of 55 mg/kg BW. STZ intravenous administration and persist throughout the experiment. Features, resulted from metabolic derangement include marked hyperglycemia, polyphagia, polydipsia, polyuria and weight loss.

1.1 Body Weight (BW)

In present study, diabetic rats (STZ) lost of their body weight compared to the non-diabetic control rats (CON). Eight weeks after STZ injection body weight were 39% lower in STZ-rats compared with non diabetic control rats ($p<0.001$) and up to 51% in 36 weeks. STZ is a compound that it had highly specific cytotoxic action on the β-cell of Islets

of Langerhans and impairs glucose oxidation through the decreases in insulin biosynthesis and secretion (Nukatsuka M et al.,1990). With a deficiency of insulin, the metabolism was then shifted from insulin-promoted anabolism to catabolism of proteins and fats, therefore, it trends to induce a negative energy balance, resulting in weight loss.

Interestingly, the data of body weight shown in Table 3. and Figure 12. demonstrated that vitamin C supplemented-diabetic rats significantly improved their body weight but only on 36 wk. This unpredicted result was also demonstrated by Paolisso G et al. (1994) suggested that the beneficial effect of an acute rise in plasma vitamin C was its capability to increase insulin action.

1.2 Blood glucose and HbA_{1C}

In present study, blood glucose levels were significantly elevated in STZ rats (397.12±65.48 mg/dl to 463.37±106.52) compared with non diabetic control rats (83.5±11.86 to 100.9±11.51; $p<0.001$). The data of plasma glycosylated hemoglobin was significantly elevated in STZ rats (9.52±1.66 to 10.13±1.88%) compared with non diabetic control rats (3.91±0.15 to 4.51±0.58 %; $p<0.001$) in all five monitored time points. The data of plasma glycosylated HbA_{1c} (Table 5. and Figure 14.) reflected long-term exposure to high blood glucose levels, to which diabetologists use as an index for how well of glycemic control (Cerami A et al., 1979).

The present study demonstrated that the supplementation of vitamin C was able to reduce plasma glucose, however, significant only at 36 weeks of treatment. These findings agree with those reported by Jariyapongskul A et al., (2002). This unpredicted result might be explained by the beneficial effect of an acute rise in plasma vitamin C on increasing insulin action (Paolisso G et al., 1994). In addition the increased plasma

vitamin C with a simultaneous increased in plasma GSSG/GSH ratio could enhanced the glucose transport. Since the intravenous injection of streptozotozin, 55 mg/kg BW, normally does not damage the whole β cells, therefore, the small amount of plasma insulin can be detected in streptozotozin diabetic rats. In other word the STZ-rat model basically represents for hypoinsulinemic state. According to the finding of Paolisso (1994), our data on hypoglycemic effects of vitamin C may be explained through the possible mechanisms of vitamin C on reducing blood glucose via its scavenging free radicals result, by which the simultaneous plasma GSSG/GSH ratio was increased, and, then increase in insulin action which the consequence of decreased plasma glucose level.

In addition, the glycosylated hemoglobin in diabetic rats was found to be significantly decreased in 36 weeks of STZ-Vit C as well. Therefore it further confirmed the benefit effect of vitamin C on reducing plasma blood glucose.

1.3 Plasma Vitamin C

In this study, the plasma vitamin C in diabetic rats was chronologically monitored by using a specific enzymatic spectrophotometric method with the absorbance at 593 nm (Liu TZ et al., 1982). The findings demonstrated that plasma vitamin C was significantly reduced in STZ-rats (23.01±0.92, 21.47±1.87 and 15.95±2.02 μmol/L; respectively) as compared those of control rats (44.59±2.12, 43.58±1.19 and 44.89±2.93; respectively). The metabolism of vitamin C has become abnormal in diabetes. And this finding supports the previous study of Kashiba M et al. (2002). They demonstrated that plasma vitamin C concentrations as well as in tissues were decreased in STZ-induced diabetic rats. The mechanism which is responsible for the matter might be due to

the competitive inhibition between vitamin C and glucose molecules. Since, transport of vitamin C through biological membrane is facilitated by diffusion through the ubiquitous glucose transporter proteins (GLUTs) (Welch RW et al., 1995). GLUTs is normally facilliatate dehydroascorbic acid (DHA) diffusion into the cell (Figure 26.). Therefore, hyperglycemic condition will be downregulated the V_{max} of the uptake mechanism of DHA. Besides, plasma vitamin C was also depleted through its anti-oxidant property; scavenging with hyperglycemia induced free radical. By means of these reasons, both plasma and intracellular vitamin C levels were reduced in diabetic state.

Vitamin C supplementation can improved the plasma vitamin C concentration in all three monitored time points of STZ-Vit C rats (43.66±3.92, 39.44±2.04 and 38.65±2.02; $p < 0.01$). The data of plasma vitamin C show in Table 6. and Figure 15.

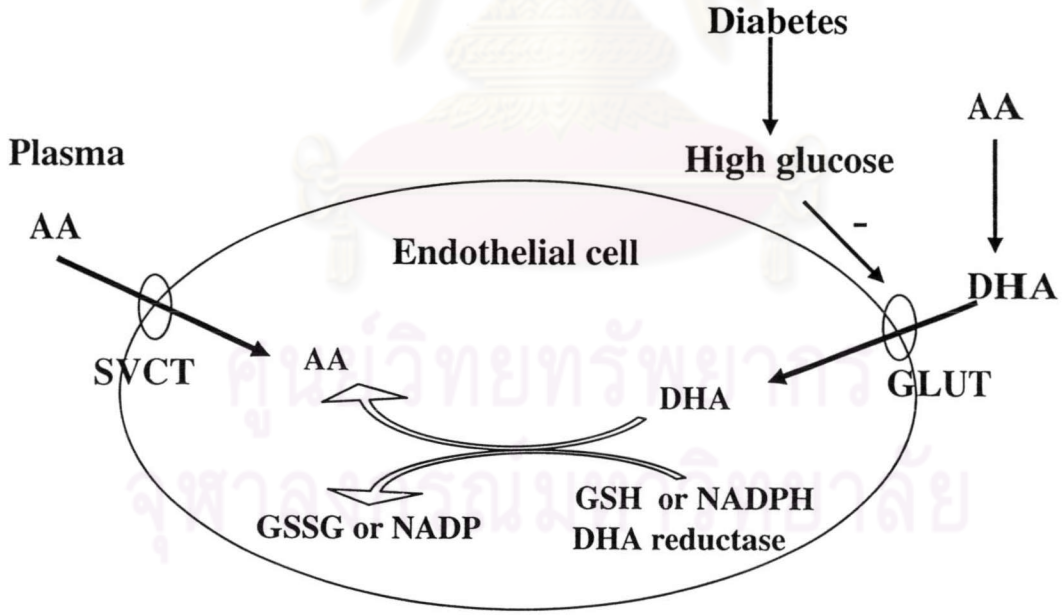

Figure 26. Scheme illustrating the major mechanism for vitamin C uptake, (Modified from Ng LL et al., 1998).

The recycling involves DHA. DHA is generated from ascorbic acid (AA) in extracellular fluid due to oxidative stress. DHA is more rapidly transported than ascorbate and is immediately reduced intracellularly to ascorbate. DHA can be converted to AA by NADPH-dependent thioredoxin reductase or glutathione- dependent DHA reductase. The reduction of DHA to AA by intracellular enzymes keeps the cytosolic concentration of DHA low and thus contributes to gradient favoring DHA uptake across the plasma membrane. It is clear that Na^+-ascorbate cotransporters (SVCT) and facilitative glucose tranporters (GLUTs) can be regulated independently. In diabetes, an excess of glucose may impair DHA uptake into cell types where DHA transport is mediated largely by facilitative glucose transporters. Therefore, there is a significant decrease in intracellular DHA and AA by mean of this high glucose competitive inhibition.

1.4 Malondialdehyde (MDA) level in eye

Lipid peroxidation is initiated by the attack free radical on fatty acid or fatty acyl side chain. Any chemical species that has sufficient reactivity to abstract a hydrogen atom form a methylene carbon in the side chain. Malondialdehyde (MDA) is generated by both lipid oxidation and as a by – product of prostaglandin and thromboxane synthesis. Its plasma concentration is increased in diabetes mellitus and it is found in the atherosclerotic plague deposits promoted by diabetes as well (Kume S et al., 1995). In our study, the MDA values were significantly higher in 8, 12, 16, 24, and 36 weeks of STZ-rats eyes (103.32±25.28, 107.11±35.75, 146.26 ±25.73, 137.59± 37.88 and 145.63±28.80) than in 8, 12, 16, 24, and 36 weeks of control rats (58.36±27.76, 72.95±21.41, 69.85±23.64, 70.49±31.39 and 73.65±27.91; respectively). These concentrations are increased in diabetes mellitus where as it is that hyperglycemia can

accelerate lipid oxidation (Niskanen LK et al., 1995). Santini SA (1997) indicated the increased lipid hyproperoxides (ROOH) and also its conjugated diene plasma levels in IDDM patients. Losada M (1997) reported that patients with retinoathy showed significantly increased MDA level compared to diabetics without retionopathy and healthy controls, using the TBA test. The degree of reactive oxygen species (ROS) occurred could be estimated by the assessment of its main product, malondialdehyde or MDA (Halliwell B et al., 1993).

Currently, there is a great interest in the potential contribution of increased oxidative stress to the development of complications in diabetes. Increased presence of ROS has also been implicated in the pathogenesis of IDDM (Santini SA, 1997). It has been suggested that long-termed exposure of body tissues to elevate blood glucose can result in diabetic patients suffering from oxidation (Wolff SP et al., 1987). Oxidative stress can produce major interrelated derangement of cell metabolism, leading to the peroxidation of cellular membrane lipids as well as the increased oxidative modification of amino acids and DNA (Halliwell B et al., 1991).

There are many suggestions regarding the origins of oxidative stress in diabetes, including free radical reactions related to glycation of proteins, consumption of NADPH through the polyol pathway, glucose autoxidation, hyperglycemia – induced pseudohypoxia, and activation of protein kinase C (Vander jagt DJ, 2000). From the result of our studies, hyperglycemia leads to non-enzymatic glycosylation of proteins, HbA_{1c}, was found to increase in STZ-rats. The serum levels of MDA correlated best with glycosylated haemoglobin. Increased lipid peroxides suggesting increased free radical activity is associated with pathogenic implications. Under normal circumstances the extent of lipid oxidation is largely controlled by antioxidant concentration in the surrounding medium which is usually sufficiently high to prevent propagation of oxidative free radical reactions

by oxygen-derived free radicals in blood. In tissue, there is, however, a greater likelihood that localized deficies of antioxidants would allow lipid oxidation to occur. (Rimm EB et al, 1993). This has led to a huge interest in dietary antioxidants and their protective role in diabetic complications.

Interestingly, vitamin C supplementation can reduce MDA values in 16, 24, and 36 weeks of STZ-Vit C rats (84.9±13.93, 81.70±21.39 and 80.88±20.84; respectively) compared with STZ-rats (146.26±25.73, 137.59± 37.88 and 145.63±28.80 ; respectively). We present evidences that continued vitamin C supplementation in diabetic rats revealed a highly significant reduction in MDA levels.

Vitamin C is the most important water-soluble antioxidant and has the potential to scavenge the superoxide and hydroperoxyl radicals, which are typical physiological forms of ROS. It has been documented for its effective scavenging superoxide and other reactive oxygen species and protection lipid against peroxidation. Several studies show that dietary antioxidants, vitamin C, can prevent propagation of oxidative free radical reactions (Beyer RE, 1994).

II. The antioxidant effects of vitamin C supplementation on leukocyte-endothelial interaction

In our experiment, the exhibition of marked enhancement of leukocyte adhesion and transmigration was observed through intravital fluorescence microscope by using rhodamine 6 G (R6G, Sigma Co.,USA) to label leukocyte.

In the present study, the iris postcapillary venules with the diameter of 20-50 μm were chosen for consequently observing the leukocyte-endothelium interaction using an intravital fluorescence microscopy on 8, 12, 16, 24 and 36 weeks after STZ injection. Our results demonstrated that

the density of leukocyte adherence per field of view was significantly increased in STZ-rats at all monitored time compared with control rats.

The peripheral polymorphonuclear leukocyte (PMN) is one of the main inflammatory cells. PMN-EC adherence resulted in the formation of a microenvironment between the PMN and the EC (Harlan JM, 1987). Under this scheme, adhesion occurs between mutually "activated" leukocytes and endothelium particularly in the small vessels and caused capillary obstruction and occlusion. The harmful of these phenomenon is that contact of circulating leukocytes with the vascular endothelium promotes a cascade of events leads to further leukocyte activation. Once activated, PMNs release ROS and mediator of proteolytic tissue degradation, contributing to oxidative stress, subsequent inflammation, and causing surrounded endothelial cells even more damaged (Smedly LA et al., 1986).

Recent studies indicate that both glucose (Schroder S, 1991; Kim JA et al., 1995), and advanced glycation end-products may damage endothelium and cause the expression of adhesion molecules (Baumgartner-Parzer SM et al., 1995 ; Schmidt AM et al., 1995). Non-enzymatic glycation of proteins may also interfere with leukocyte behavior. Masuda M et al., (1990) have demonstrated that glycosylated protein separated from the serum of diabetic rats is capable of decreasing membrane fluidity of control leukocyte which may alter leukocyte function such as leukocyte migration. Even a small reduction in leukocyte deformability would likely increase leukocyte retention in capillaries. The observation of less deformable leukocytes has also been extended to both IDDM and Type II diabetic patients (Ernst E and Matrai A, 1986 ; Pecsvarady Z et al., 1994). Sannomiya P et al. (1997) demonstrated that aminoguanidine, an inhibitor of advanced glycation end products formation, prevented the decreased leukocyte rolling and migration in alloxan-diabetic rats.

In the present study, vitamin C supplementation had the effect to reduce the number of leukocyte adhesion to endothelium of postcapillary venule in 24- and 36-week STZ-Vit C rats, but not equal to the control values. Zanardo RO et al. (1998) have previously demonstrated that vitamin C corrected the reduced cell migration in alloxan–diabetic rats as well. Raised levels of leukocyte adhesion have been demonstrated in diabetic rats. Besides, it had been prognostic importance in the development of diabetic complications. For instance, the role of leukocytes in the pathogenesis of proliferative diabetic retinopathy has been suggested (Schroder S, 1991; Forrester JV, 1993). Especially, greater leukocyte adhesion in diabetic venules was able to reason by dysfunctional diabetic endothelium. Because of several studies suggested that nitric oxide (NO) was reduced in the endothelium of diabetic vessels. In addition, the effects of NO not only on vascular smooth muscle, but also Kubes P et al. (1991) reported that inhibition of nitric oxide production resulted in a 15-fold increase in leukocyte adherence to cat mesenteric venules. Additionally, NO was also reported to reduce leukocyte adhesion in an acute model of canine myocardial ischemia and reperfusion (Lefer DJ et al., 1993). The increased oxidative stress due to hyperglycemia has been reported for its effects on decreasing nitric oxide activity and synthesis (Jariyapongskul A et al., 2002, Sridulyakul P et al., 2003). From our all results, therefore, we suggested that the increase in generation of oxygen – derived free radicals (demonstrated by MDA levels) is the major contributors to induce increasing leukocyte adhesion in diabetic rats. Since ROS might cause the decrease in NO, therefore, the activation of adhesive molecule were occurred. Through this matter, it explains the reason why could improve or prevent leukocyte adhesion.

III. The antioxidant effects of vitamin C supplementation on regional iris blood-flow perfusion

By using laser droppler flowmetry, mean regional iris blood-flow perfusion of STZ rats was monitored as described in Chapter III. The results showed that iris blood-flow in STZ-rats was significantly reduced by 33.64 %, 50.46 %, 57.95, 57.60 % and 56.12 compared with 8, 12, 16, 24, and 36 weeks control rats, respectively.

Interestingly, while iris-blood-flow perfusion was become significantly decreased since 8-week period, it seems to have the trend of hypertension. Because of systolic and diastolic blood pressure (SBP, DBP) monitored at 16-36 wks were significantly increased as compared to their eye controls. In present study demonstrated that the supplementation of vitamin C was significantly decreased SBP and DBP at only 24, 36 weeks, not different compare with control rats in 8, 12, 16, 24 and 36 week. Ascorbic acid is an extremely potent free radical scavenger and may thus protect nitric oxide from excessive degradation. As which it can prevent endothelium dysfunction therefore, it can prevent the initiation of hypertension.

Therefore, we believed that the abnormality of iris-blood perfusion occurred as early as 8-weeks diabetic duration could not simply explain by the systemic circulation, but by diabetic microangiopathy. Kawagishi T et al. (1995) showed that changes in retinal hemodynamics were present ed before the clinical detection of overt diabetic retinopathy, therefore they suggested that the presence of short-term hyperglycemia partly contributed to impair retinal microcirculation. However, the diverging results was showed by Tilton et al who reported that retinal blood flow in streptozotocin induced diabetic rats increased within 6-weeks duration. Normally, the blood-flow perfusion of iris depends on hemodynamic and

vascular factors in the carotid. The hemodynamic changes may contribute to the development of morphological abnormalities. The possible abnormalities might be due to the changes level of in the vasculature; such as basement membrane thickening (Tilton RG et al., 1981) and pericyte loss. Pericytes are very important in the regulation of blood flow since they possess contractile properties and allowing the changes of the vessel lumen diameter (Takahashi K et al., 1989). In addition, changes in the rheological properties of blood, such as abnormalities of fibrinotic response (Almer LO et al., 1976 ; Lowe GDO et al., 1986), increased platelet aggregation (Mc Millan DE, 1983 ; Juhan I et al., 1982), increased blood viscosity, decreased red cell deformability (Mc Millan DE, 1983; Juhan I et al., 1982) and decreases in erythrocyte and leukocyte deformability (Mc Millan DE, 1978 ; Schmid – Schonbein G et al., 1976) all which occurred in diabetes mellitus, could also contribute to the changes in blood flow. These lead to decrease capillary blood flow and even thrombosis in the smallest vessels of the iris.

Several studies have also reported that hyperglycemia can increase the expression of vasoconstrictors, such as endothelin-1 (ET-1) and angiotensin or their actions (Takagi C et al., 1996). The over expression of vasoconstrictors that have been associated with increased vascular resistance and decreased iris blood-flow perfusion. Tooke JE (1986) postulated that increased glycation and thickening of the basement membrane result in changes of vascular structure. This rigid, therefore, increases resistance to flow and causes an increase in shear stress.

In our study, microaneuryms (Figure 27.) were able to observe especially 24 and 36 weeks. It was suggested that the distension of the vascular wall at sites of microaneurysm formation may be resulted from the lack of arterial control of blood flow in which it has already compromised by loss of smooth muscle cell or pericytes.

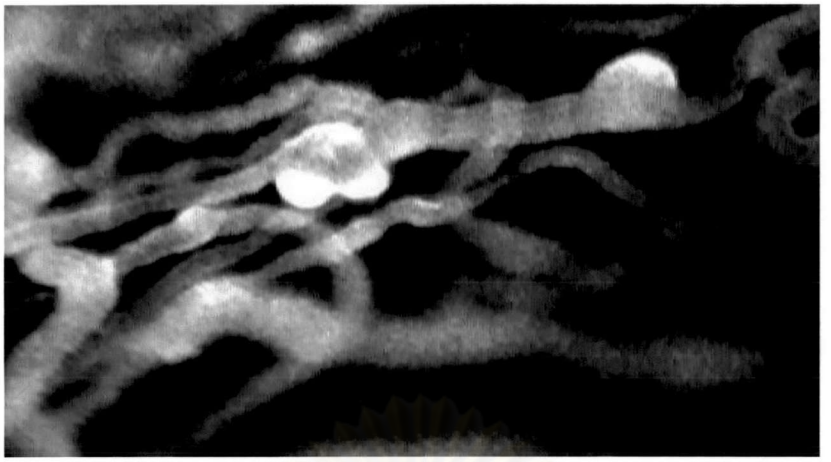

Figure 27. Demonstrate the abnormality of arteriolar vascular wall. Microaneurysm was observe only in occurred in the group STZ. However the kind of morphological change in STZ-Vit C.

Our present study demonstrated that the supplementation of vitamin C was significantly increased iris blood-flow perfusion at 24 and 36 weeks. Interestingly, our results have demonstrated that the reduced iris blood-flow perfusion could be prevented by vitamin C supplementation. The mechanisms for the ability of ascorbate to enhance endothelium-dependent vasodilation has been demonstrated both by intravenous ascorbate infusions (Kugiyama K et al., 1998 ; Solzbach U et al., 1997 ; Tousoulis D et al., 1999) and by oral ascorbate supplementation (Levine G et al., 1996 ; Gokce N et al., 1999, Jariyapongskul A et al., 2002). Whereas the ascorbate infusion studies generated very high local plasma ascorbate concentrations (up to 10mM ascorbate), the results of the oral supplementation studies suggest that these effects of ascorbate can be achieved even by the with physiologic concentrations. In accordance to the experimental data of leukocyte and iris perfusion, it may be summarized and proposed for the hypothesis showed in the following diagram (Figure 28.). The idea is that

the hyperglycemia induced reactive oxygen species (ROS) to increase, in which it can be demonstrated by the increase in MDA level. Because of ROS, the endothelium was become dysfunction, therefore, it increased leukocyte-endothelial interaction, and consequently resulted in an enhancement of iris blood-flow perfusion to decrease. By which role of vitamin C as an antioxidant, therefore, it decreased ROS as then resulted to the prevention of endothelial dysfunction.

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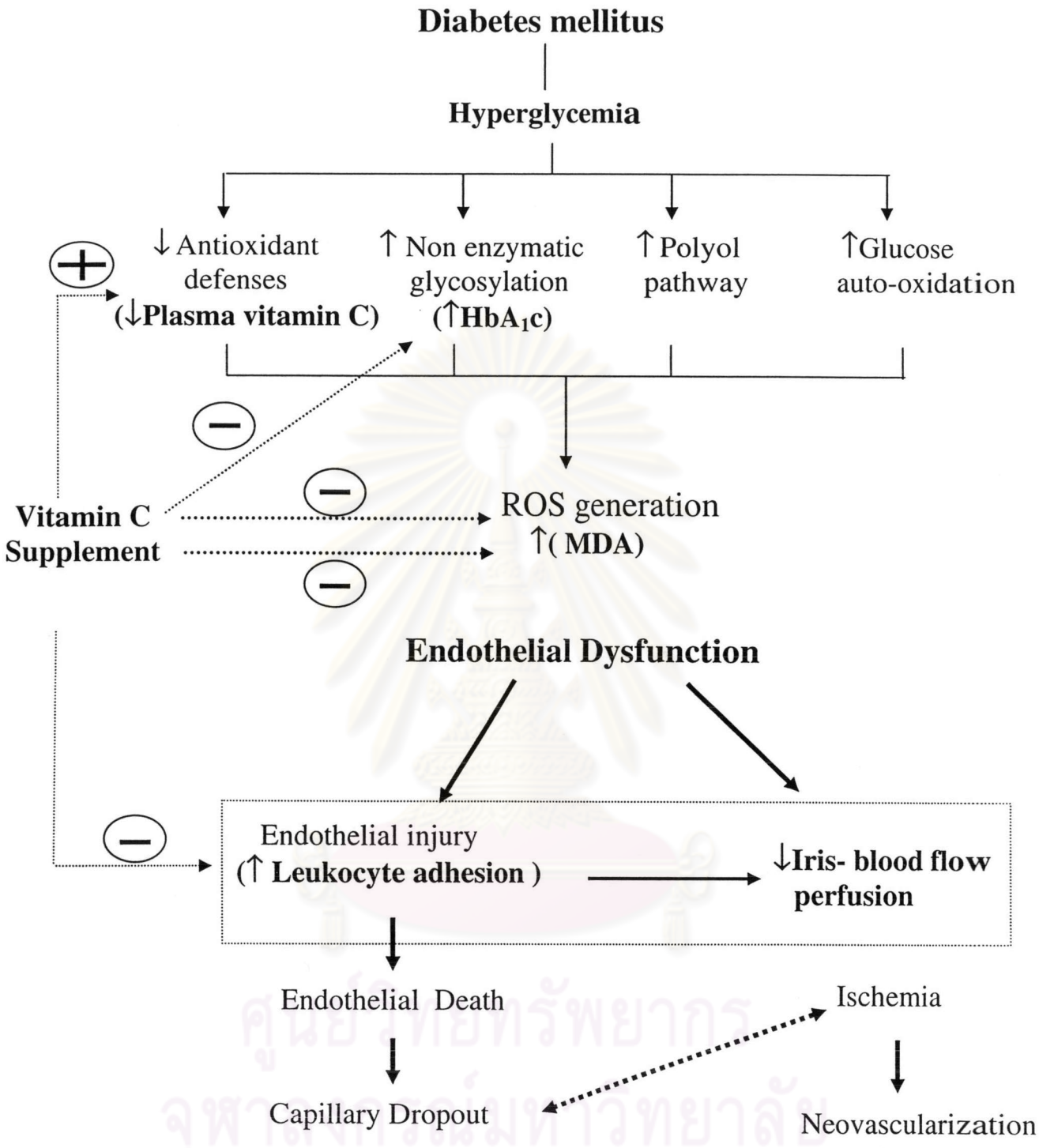

Figure 28. This diagram demonstrates the proposed mechanism for which vitamin C can effect diabetes induced endothelial dysfunction.

IV. Relationship on iris blood-flow perfusion and leukocyte endothelial cell interaction

One of our major objective was to determine whether the increased leukocyte adhesion were contribute to the decrease in iris blood-flow perfusion or not. The increased leukocyte adhesion to the capillaries have been noted to occur in diabetic patients and diabetic animal model. In addition, those findings have been suggested to the important for the development of diabetic angiopathy, since both reduced blood flow and increased leukocyte adhesion may contribute to the formation of nonperfused capillaries, which are believed to be major contributors for the progression of diabetic angiopathy and result to the with increase in capillary permeability and angiogenesis.

In our study, we have identified both two changes of iris blood-flow perfusion and leukocyte adhesion as early as 8 weeks of diabetic induction. In addition, the result also indicated that increased leukocyte adhesion was correlated to the decrease in iris blood-flow perfusion significantly. As which the correlation between leukocyte adhesion and iris blood-flow perfusion can be characterized by the linear regression showed in Figure 24., $y = -0.447x+32.80$, $r = -0.317$, $p < 0.034$. Interestingly, the correlation was more clarified and supported by the results of STZ-Vit C, $y = -1.862x + 47.103$, $r = -0.517$, $p < 0.001$. Several reports have suggested that increase in leukocyte / monocyte adhesion was a critical factor in early retinopathy causing decrease in retinal blood flow and increase in cytokine expression, as well as vascular endothelial growth factor (Miyamoto K et al., 1999; Joussen AM et al., 2002). Miyamoto K et al. (1999) reported that increased in leukostasis been shown in animal models of diabetes with short duration of disease. Multiple reports have suggested that the increase in leukostasis was associated with leukocyte or endothelial cell activation and increased

expression of adhesion molecules on both cell types (Miyamoto K et al., 1999; Lo SK et al., 1993).

Accordingly, the results demonstrated the correlation between increased leukocyte adhesion and decrease in iris blood–flow perfusion was significantly characterized by the linear regression. Therefore, would like to hypothesize the simple reason explained such correlate as that once hyperglycemia induced endothelial dysfunction, may be through the decrease of nitric oxide activity, as described previously therefore, more adhesive molecules of both leukocyte and endothelial cell were expressed. As when leukocyte adhesion occluded midperipheral vessels then large areas of capillary nonperfusion were developed and finally it may contribute to hypoxic development as a consequence of ischemia. The more leukostasis causes the vascular occluded, the more capillaries become no flow, and results to less of iris blood-flow perfusion (Figure 29).

The data demonstrates that the presence of diabetes are necessary for measurable effect on iris blood-flow perfusion can occur in diabetes, not founded relationship in control rats. Because of the supplementation of vitamin C could prevent both the increase in leukocytes adhesion and decreases in iris blood-flow perfusion in diabetic rats. By mean of this linear correlation $y = -1.8627x + 47.103$, $r = -0.517$, $p < 0.001$. One might simply say that the prevention of leukostasis probably via the inhibition of expression of adhesive molecule (Rayment SJ et al., 2003) may be the therapeutic tool for preventing hypoxic condition in diabetic iris including retina.

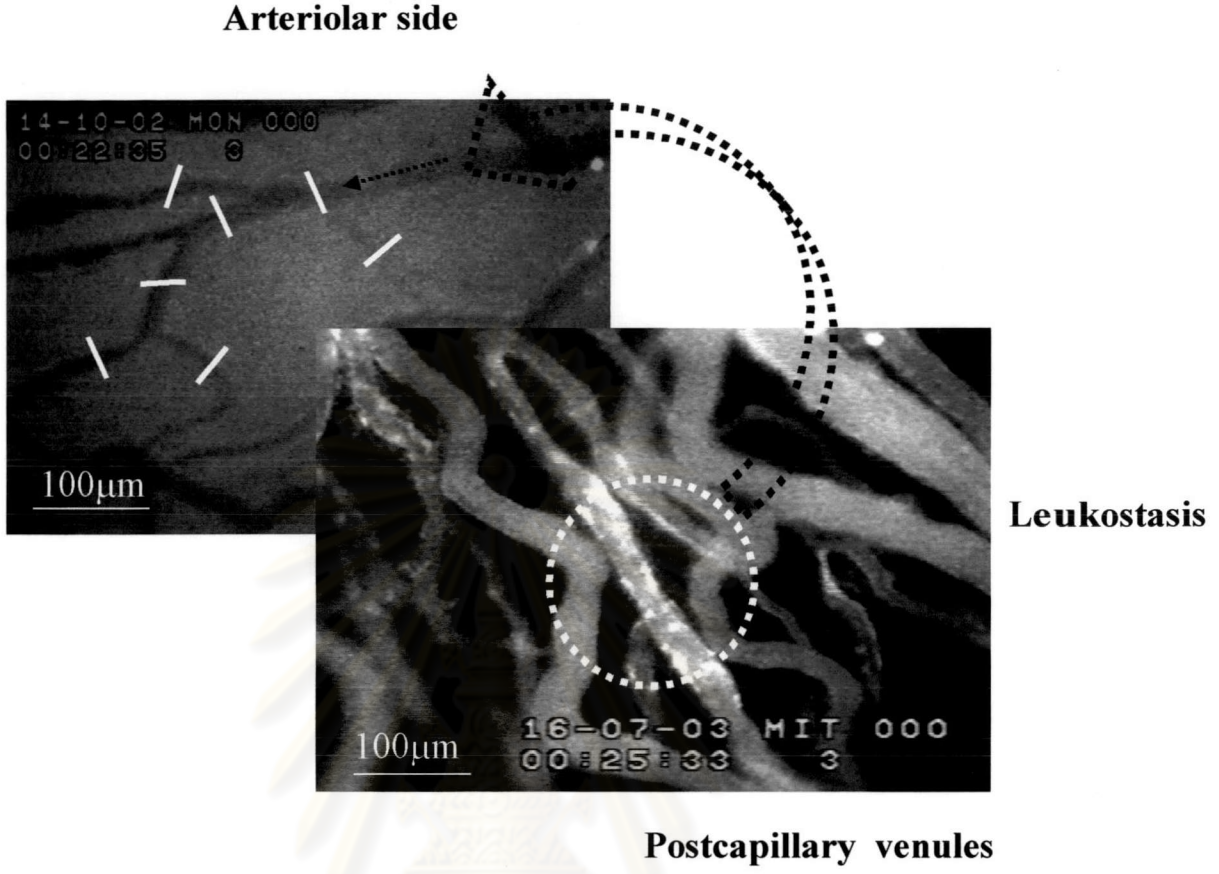

Figure 29. Hypothesis for explaining the relationship between iris blood-flow Perfusion and leukocyte adhesion. Because of leukostasis occluded postcapillary venules, therefore, the pressure gradient was dropped. The inlet-flow was then declined down in accordance to their dropped pressure gradient. And this might bring about the reason why iris blood-flow perfusion was decreased when the number of leukocyte adhesion increased.