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Original article

Leukocyte-endothelial cell interaction is attenuated by low-intensity exercise training and vitamin C supplementation in diabetic rats

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Objective: To determine the effects of vitamin C supplementation and low-intensity exercise training on diabetes-induced endothelial dysfunction.

Methods: Male Spraque-Dawley rats were randomly divided into five groups: control (Con), diabetes (DM) (streptozotocin; 50 mg/kg BW, i.v.), diabetes with supplemented vitamin C (DM+Vit.C; 1 g/L mixed in drinking water), diabetes with low-intensity exercise-trained (DM+Ex; running 5 times/week with 13-15 m/min velocity for 30 minutes) and diabetes with supplemented vitamin C and exercisetrained (DM+Vit.C+Ex) groups. The number of leukocyte-endothelial cell (EC) interactions in mesenteric postcapillary venules was monitored using intravital fluorescence videomicroscopy. Liver malondialdehyde (MDA) level, an indicator for oxidative stress, was determined by using the thiobarbituric acid reaction.

Results: At 24 weeks, the plasma vitamin C level was significantly increased ($p<0.05$) in DM+Vit.C and DM+Vit.C+Ex rats when compared with DM rats. DM+Ex and DM+Vit.C+Ex rats had lower triglyceride levels and heart weights when compared with DM rats ($p<0.05$). Mean arterial pressures were significantly decreased in all treatment groups. DM rats had significantly higher malondialdehyde (MDA) levels and lower activities of superoxide dismutase (SOD) than Con. The number of adherent leukocytes and levels of MDA were significantly lower in DM+Vit.C, DM+Ex and DM+Vit.C+Ex than those of DM rats.

Conclusion: The increased leukocyte-EC adherence in diabetic rats is significantly related to increased ROS, based on lower MDA levels. Vitamin C supplementation and regular low-intensity exercise training can prevent these deleterious effects, including hypertension, cardiac hypertrophy, hypertriglyceridemia, and leukocyte-EC adherence. Vitamin C supplementation combined with low-intensity exercise training is highly effective in preventing diabetic cardiovascular complications.

Keywords: Diabetic rat, endothelial dysfunction, exercise training, leukocyte, vitamin C.

Endothelial dysfunction has been documented as the key process of both macro- and micro-angiopathy in diabetes mellitus. The development of endothelial dysfunction can be characterized by both impairment of vasorelaxation and increased expression of adhesion molecules. The increase in reactive oxygen species (ROS), subsequently produced from long-term hyperglycemia, was suggested as a major underlying cause of diabetic-induced endothelial dysfunction [1-3]. The interaction between leukocytes and endothelium is one of the markers for diabetes-induced endothelial dysfunction. Interestingly, our previous studies in diabetic rats have shown that vitamin C supplementation could reduce leukocyte-endothelial cell interactions in both experimental models of long-term preventive and early phase reversal trials [4, 5].

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Recently, the combined effect of vitamin C and a hypolipidimic agent, like curcumin, has been shown to have more beneficial effects on endothelial function in diabetes-induced ROS [6]. ROS within the vasculature may initiate a vicious cycle by oxidizing lipid or lipoprotein (a) which in turn may cause endothelial dysfunction. There is also growing evidence that exercise training may have favorable effects on oxidant and anti-oxidant status, in particular on decreasing lipid peroxidation [7-11].

Based on these findings, the present study was aimed to determine whether low-intensity exercise training combined with vitamin C supplementation is able to prevent the diabetes-induced endothelial dysfunction, characterized by the interaction between leukocytes and endothelium, by balancing oxidative and anti-oxidtive systems.

Materials and methods

Animal preparation

Male Spraque-Dawley rats, weighing 200-250 g, were used in this study. All rats were purchased from National Laboratory Animal Center and cared in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care, U.S. Department of Agriculture, and the National Research Council. All rats were divided randomly into five groups: control (Con), diabetes (DM), diabetes with supplemented vitamin C (DM+Vit.C), diabetes with exercise training (DM+Ex), and diabetes with supplemented vitamin C and exercise training (DM+Vit.C+Ex). The experimental protocol was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University.

Diabetes induction

To induce diabetes mellitus, streptozotocin (STZ) (Sigma Chemical Co. USA) was freshly prepared by dissolving in citrate buffer pH 4.5 (Sigma Chemical Co. USA) and injected immediately into the tail vein of fasted rats (50 mg/kg body weight) under general anesthesia induced by 60 mg/kg body weight of sodium pentobarbital (intraperiotneally.). Two days after the STZ-injection, blood glucose was determined by using a glucometer (Advantage Glucometer, Boehringer Mannheim, Germany). At 48 hours, STZ-rats that failed to exhibit an elevation of blood glucose level greater than 200 mg/dl, were excluded from the study [4].

Vitamin C supplementation

Supplementation of vitamin C (L-ascorbic acid, 99 %, Sigma USA) was started 48 hours after administration of streptozotocin. Vitamin C was prepared daily by dissolving it in drinking tap water at a concentration of 1 g/L; the experimental rats had free access to this vitamin C drinking water [4].

Exercise training protocol

A low-intensity exercise training protocol, modified from the studies of De Angelis et al [12] and Wang et al [13], was used. Briefly, the 24-wk exercise protocol consisted of running on a motorized treadmill (SPORTSART 1190) five times per week and 30 min per day. During the 30-min exercise, the maximum level of activity, 15 m/min was performed with 5-min warm up and 5-min warm down at 13 m/min and 0° grade setting. The training was performed after 5 pm to ensure that the exercise took place in the rat daily active cycle.

Study of mesenteric microcirculation

The experiments were performed at 24 weeks after the STZ injection. On the day of the experiment, the rats were anesthetized by intraperitoneal injection (i.p) of 45 mg/Kg.BW of sodium pentobarbital. After the tracheostomy, polyethylene catheters were inserted into the left common carotid artery (PE 90) and the left external jugular vein (PE 20) for monitoring arterial blood pressure and heart rate during intravenous drug administration, respectively. Mean arterial pressure (MAP) and heart rate (HR) were monitored and recorded throughout the experimental period by using a polygraph system ((NIHON KODEN, Japan).

After the loop of mesentery was exteriorized through a midline incision and placed on a plexiglass chamber, Krebs Ringer Solution (pH 7.4) at 37°C was infused continuously. The mesenteric micro-circulation of the distal ileum was observed under an epi-illumination fluorescence video microscope (Nikon, Optiphot-2 model, Japan) with a 50-W mercury lamp and a fluorescence filter.

At the end of each experiment, the blood sample of each rat was collected for determination of blood glucose (BG), lipid profiles, and glycosylated hemoglobin (HbA_{1C}) by RIA Laboratory Co, Ltd. (Bangkok, Thailand).

Leukocyte-endothelial intreaction

After 15-20 min stabilization, a single unbranched mesenteric postcapillary venule (20 to 45-μm diameter, >150 μm length) was chosen for each observation. Acrydine orange at concentration of 25 mg/ml was intravenously injected as a bolus (0.5 ml) via the canulated jugular vein. By using fluorescent microscopy, video recordings were used for quantification of leukocyte adherence. The equipment set consisted of video microscope (Nikon, Tokyo, Japan) with a 40x objective lens (Nikon, Japan), video camera (MTI -SIT68), video recorder (Sony GUM-1411QM, Japan), video timer (Sony, Japan), and video printer (Sony video graphic printer UP-890CE).

Numbers of adherent leukocytes were analyzed off-line by using Global Lab Image Software (Data Translation, MA, USA). A leukocyte was considered to be adherent to postcapillary venule (15-30μm) if it remained stationary for 30 seconds. The number of leukocyte adherences was expressed as the number of cells per 100-μm postcapillary venule length [4].

Oxidant and antioxidant analysis

After intravital video microscopy, the liver of each animal was excised and immersed in an ice-cold 0.9% NaCl. Fat and fibrous tissue was removed before weighing. The livers were collected for measurement of malondialdehyde (MDA) level and superoxide dismutase (SOD) activity. MDA level was determined using the thiobarbituric acid reaction as described by Ohgawa et.al [14]. SOD activity was determined by the modified method of Winterbourn et al [15].

Data analysis

All data are presented as means and standard errors of mean (SEM). For comparison among groups of animals, one way analysis of variance (one-way ANOVA) was used and the differences in pairs of means among groups were made by Turkey's test. Pearson's correlation was used to investigate relationships between number of leukocyte and MDA. If the statistical probability (p-value) was less than or equal to 0.05, differences between means were considered to be statistical significant.

Results

Physiological characteristics

The results shown in **Table 1** demonstrated that the low intensity exercise training program used in our study (running on a motorized treadmill 15 m/min, 30 min/day, and 5 days/week) for 24-wk experimental periods, was able to decrease heart rate, heart weight, and mean arterial blood pressure (MAP) indicated in both the DM+Vit C+Ex and DM+Ex ($p<0.05$) groups. MAP of DM+Vit C was significantly less than DM at 24 weeks ($p<0.01$). There was no significant difference in blood glucose and HbA_{1C} between DM and DM+Ex rats. Plasma vitamin C levels of 24-wk DM and DM+Ex were two-fold less than the mean level of Con ($p<0.05$).

Our findings demonstrated that triglyceride (TG) levels were significantly increased during 24 weeks of diabetes ($p<0.001$), whereas there was no significant difference for other lipids between DM and Con groups. In addition, the results indicated that long-term exercise training and treatment with vitamin C can attenuate hypertriglyceridemia, as shown by mean TG levels in the 24-wk DM+Ex group compared with DM ($p<0.01$).

Oxidants/antioxidants analysis

In this study, malondialdehyde (MDA) was used as an indicator of oxidative stress. Data shown in **Table 1** indicate that MDA levels of the 24-wk DM liver homogenate were significantly (about 2-fold) higher than Con ($p<0.001$). The MDA level of the 24-wk DM+Ex was significantly lower ($p<0.01$) than DM; however, there was no significant difference between mean values of MDA in the 24-wk DM and DM+ Vit C groups.

Leukocyte adhesion to endothelium

By using image software analysis, the number of leukocytes adherences per 100 μm of vessel length were obtained from each group. The results of leukocyte adherence were increased by 2-fold in 24-wk DM as compared with Con ($p<0.01$; Table 2). Interestingly, the number of leukocyte adherences were significantly reduced in DM+Ex, DM+ Vit C, and DM+Vit C+Ex as compared with DM ($p<0.001$).

Discussion

This is the first long-term *in-vivo* study that reports the combined effects of low-intensity exercise training and vitamin C on leukocyte-endothelium interaction in the intact mesenteric vessels of 24-wk STZ-induced diabetic rats. Our results showed that: 1) the low-intensity exercise training adequately prevented diabetic induced hypertension, cardiac hypertrophy, and hypertriglyceridemia. 2) The

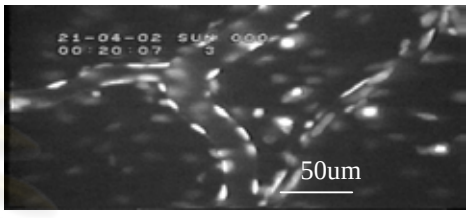
Table 1. The physiological and biochemical characteristics of control, diabetes (DM), and diabetes with exercise training (DM+Ex), diabetes with vitamin C supplementation (DM+Vit.C), and diabetes with both vitamin C supplementation and exercise training (DM+Vit.C+EX).

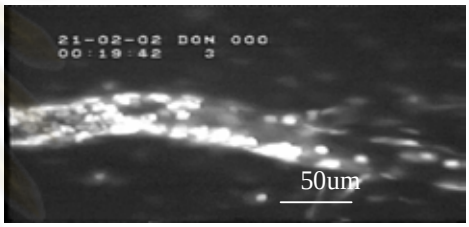
	Control	DM	DM+Ex	DM+Vit. C	DM+Vit C+Ex
Body weight (g)	489.82±10.98 (n = 11)	294.65±8.07*** (n = 10)	283.72±19.12*** (n = 9)	266.50±11.17*** (n = 9)	289.38±14.14*** (n = 8)
Heart weight (g/kg bw)	2.91±0.08 (n = 10)	4.07±0.09*** (n = 8)	3.47 ±0.11$^{\#}$ (n = 10)	4.45±0.25*** (n = 9)	3.47±0.10 (n = 7)
Heart rate (bpm)	308.33 ±8.33 (n = 9)	290.63±14.89 (n = 8)	237.71±16.54* (n = 7)	296.88±13.72 (n = 8)	238.31±19.63* (n = 7)
MAP (mmHg)	88.33±3.18 (n = 8)	119.82±6.71** (n = 9)	88.20±3.9$^{\#\#}$ (n = 8)	93.75±5.79$^{\#\#}$ (n = 10)	81.42±5.56$^{\#\#}$ (n = 8)
Blood glucose (mg/dl)	98.00 ±5.10 (n = 8)	327.67±20.82*** (n = 9)	311.88±23.17*** (n = 8)	287.80±6.47*** (n=10)	361.00±12.55*** (n=8)
Glycosylated hemoglobin (%)	3.41±0.09 (n = 8)	11.20 ±0.38*** (n = 8)	10.12 ±0.25*** (n = 9)	9.81±0.30***, $^{\#}$ (n=8)	9.75±0.44***, $^{\#}$ (n=8)
Plasma vit. C (mg/dl)	1.17 ±0.11 (n = 7)	0.51±0.04* (n = 7)	0.67 ±0.06 (n = 7)	1.58±0.14$^{\#}$ (n=8)	1.12±0.06$^{\#}$ (n=8)
Liver MDA levels (nmole/g wet wt.)	3632.14±441.61 (n = 7) 7740.09±403.18	5646.56±232.52*** (n = 10) 5311.73±195.31**	4135.98±131.53$^{\#\#}$ (n = 8) 5140.45±441.08**	5480.95±215.67 (n=10) 6132.88±435.58	4121.52±303.17$^{\#\#}$ (n=10) 6511.97±565.61
Liver SOD activity (units/g wet wt.)	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
Plasma Cholesterol (mg/dl)	86.71 ±4.44 (n = 7)	94.33±8.45 (n = 6)	96.44±6.26 (n = 9)	81.50 ±2.19 (n = 8)	97.14±9.97 (n = 7)
Plasma Triglyceride (mg/dl)	64.71±9.71 (n = 7)	150.83 ±17.71*** (n = 6)	91.50±10.50$^{\#\#}$ (n = 6)	122.25±11.35$^{\#}$ (n = 8)	93.57±14.05$^{\#\#}$ (n = 6)
High density lipoprotein (mg/dl)	55.29±2.89 (n = 7)	58.17±4.92 (n = 6)	52.88±2. 22 (n = 6)	58.11±3.56 (n = 8)	64.33±7.41 (n = 6)
Low density lipoprotein (mg/dl)	11.86±1.20 (n = 7)	13.17±0.95 (n = 6)	10.50±1.38 (n = 6)	15.56±1.46 (n = 8)	18.03±3.12 (n = 6)

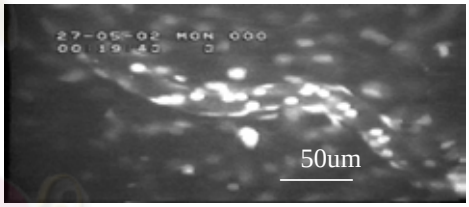
*p<0.05 significantly different compared with the control; **p<0.01 significantly different compared with the control; *** p<0.001 significantly different compared with the control; $^{\#}$ p<0.05 significantly different compared with the DM; $^{\#\#}$ p<0.01 significantly different compared with the DM; $^{\#\#\#}$ p<0.001 significantly different compared with the DM.

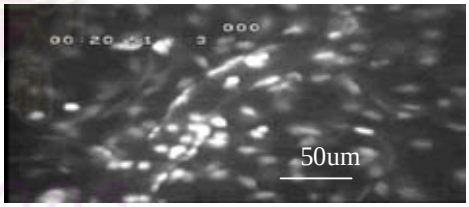
Table 2. Means±SEM of number of leukocyte adherence (cells/100μm postcapillary venule length) were demonstrated. Values and video images were demonstrated for five groups: control, diabetes (DM), diabetes with exercise training (DM+Ex), diabetes with vitamin C supplementation (DM+Vit.C), diabetes with both vitamin C supplementation, and exercise training (DM+Vit.C+EX). ** p<0.01 significantly different compared with the control; ### p<0.001 significantly different compared with the DM.

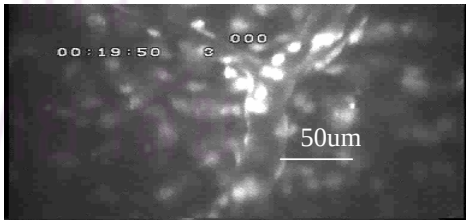
Group	The number of leukocyte adherence (cells/100μm vessel length)
Control	5.57±0.97 (n = 7)
DM	11.86 ±0.86** (n = 7)
DM+Vit.C	3.14±1.06### (n = 7)
DM+Ex	5.13±1.13### (n = 8)
DM+Vit.C+Ex	5.14±0.67### (n = 7)











increased in liver MDA levels were significantly attenuated by low-intensity exercise training (data of DM+Ex and DM+Ex+Vit C groups). 3) Exercise training did not demonstrate any significant effects on plasma glucose and HbA_{1C}, whereas HbA1c were significantly reduced by vitamin C supplementation (data shown in DM+Vit C and DM+Ex+Vit C groups). 4) The number of leukocyte-endothelial cell interactions, used as the indicator for endothelial dysfunction, could be attenuated by both low-intensity exercise training and vitamin c supplementation (data of DM+Vit C, DM+Ex+Vit C, and DM+Ex groups).

Even though the mechanisms of hyperglycemia-induced endothelial dysfunction are complex and not completely explained, there is substantial evidence indicating the role of reactive oxygen species (ROS) in mediating the vascular endothelial dysfunction in diabetes [16-20]. In the present study, the degree of ROS production was assessed based on its main product, malondialdehyde. MDA levels in liver homogenates were significantly higher in 24-wk diabetic rats.

Our results showed that DM rats had significantly lower SOD activity at 24 weeks as compared with Con. SOD is an important endogenous anti-oxidant enzyme for scavenging ROS [23, 30-31]. Evidence clearly suggests that there is an imbalance of the oxidative and anti-oxidative systems within the cell due to hyperglycemia. This could cause the increase in cellular ROS produced by mitochondrial respiratory chain. Even though, SOD did not demonstrate an increase in treated groups, our findings indicated that the MDA production was decreased in parallel with the prevention of hypertriglyceridemia in the exercise training group.

The increased production of ROS has been documented for its role in the etiology of hypertension, but the effects of antioxidants on blood pressure remain controversial. [21-23]. The present study showed that diabetes induced hypertension could be prevented by long-term treatment with vitamin C. It has been shown that chronic treatment with ascorbic acid markedly reduced blood pressure and arterial stiffness in Type 2 diabetes [22]. Moreover, the preventive effects of vitamins C and E on progression of hypertension in hypertensive rats are believed to be mediated via the modulation of enzyme systems that generate free radicals [23].

Our study also indicated that not only vitamin C but also a 24 weeks of low-intensity exercise training could significantly reduce the mean levels of arterial pressure in DM rats. In addition, our results also showed that diabetes induced-cardiac hypertrophy could be prevented. We propose that low-intensity exercise training can prevent cardiac hypertrophy by attenuating the changes of total peripheral vascular resistance, which is the underlining cause of diabetes induced hypertension.

ROS has been well documented for its involvement in mediating the decrease in endothelial nitric oxide synthase (eNOS) activity. Decrease in NO will result in activated protein kinase C (PKC) which can cause the upregulation of adhesion molecules expression [24-28]. From our findings, the number of leukocytes adhering per 100 mm of vessel length was significantly increased in 24 weeks diabetic rats compared with controls. This is in agreement with our previous reports showing diabetes-induced leukocyte-endothelium interaction in both cerebral and iris microcirculation of diabetic rats [4-29]. We would therefore like to suggest that high blood glucose levels could mediate oxidative stress, resulting in reduced NO availability, thus enhancing adhesion molecule expression and subsequently leading to enhanced leukocyte-endothelium interaction.

Interestingly, the present study has shown that the diabetes-induced leukocyte-endothelium interaction could be attenuated not only by vitamin C but also by low-intensity exercise training. Because glucose-induced leukocyte adhesion is dependent upon the upregulation of adhesion molecules including, E-selectin, ICAM-1, and VCAM-1 [24-28], our findings imply that vitamin C and low-intensity exercise training are capable of attenuating this hyperglycemic effect.

Since ROS has been implicated in potentially up regulating adhesion molecule expression, we propose that exercise training can prevent ROS induced leukocyte-endothelial cell interaction in diabetic rats by lowering oxidative stress and dyslipidemia, as demonstrated by a reduction of MDA and TG values, respectively. This molecular mechanism underlying the protective effect of low-intensity exercise training, is supported by strong correlation between MDA levels and the number of leukocyte-endothelial cell interactions, as shown in **Fig. 1** ($r = 0.74$, $p < 0.001$).

We have demonstrated that endothelial dysfunction in diabetic rats, manifested as increased leukocyte-endothelial interaction, can be prevented by vitamin C supplementation. A significant reduction in MDA levels was observed at 12 weeks after *DM+Vit C (4355.83±508.27 nmole/g wet wt.) compared to DM (6141.22±366.09 (nmole/g wet wt; p<0.05)*, but not after 24-wk DM+Vit C. Inactivation of endothelium-derived nitric oxide by reactive oxygen species such as superoxide anion (O_2) may contribute to endothelial dysfunction [31-32]. Thus, we believe that vitamin C can protect against the development and progression of endothelial dysfunction in diabetes via its competitive interaction with ROS, thus disabling ROS from interacting with NO in endothelial cells. However, the exact mechanism by which vitamin C maintains optimal antioxidant capacity in endothelial

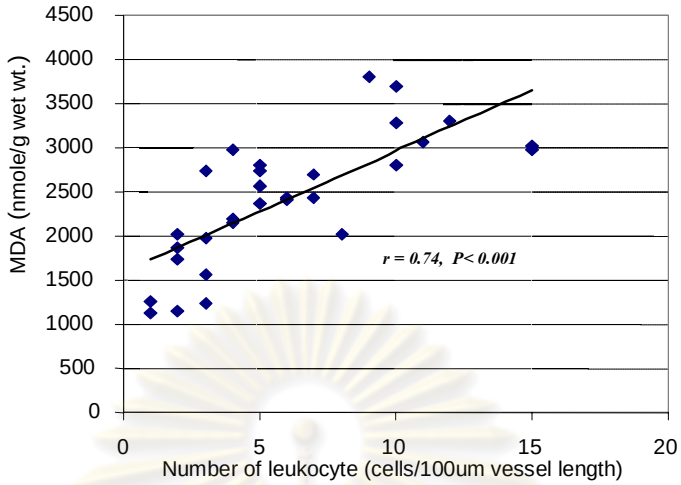


Fig. 1 The correlation between liver MDA levels and the number of leukocyte-endothelial cell interaction ($r = 0.74$, $p < 0.001$).

cells and prevents formation of the cytotoxic peroxynitrite ($ONOO^-$), still requires further study.

The results likewise show the beneficial effect of low-intensity exercise training in attenuating leukocyte adherence in 24-wk diabetic rats, similar to the effect of vitamin C alone. However, there appeared to be no synergistic effect between low-intensity exercise training and vitamin C supplementation in terms of inhibiting leukocyte-endothelium interaction (as demonstrated by the result of DM+Ex+vit C group).

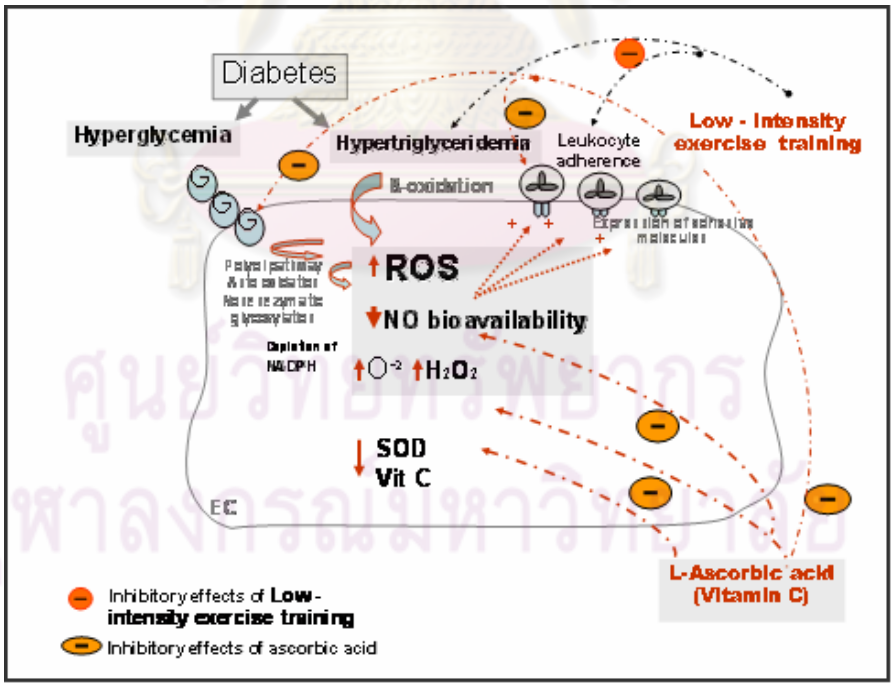


Fig. 2 Both regular low-intensity exercise training and vitamin C supplementation can prevent diabetes-induced endothelial dysfunction, in particular, the leukocyte-endothelial cell (EC) interaction. Low-intensity exercise training can decrease ROS through the prevention of dyslipidemia. Vitamin C can scavenger ROS, both superoxide (O^{-2}), and hydrogen peroxide (H_2O_2), therefore, it can increase or preserve nitric oxide (NO) bioavailability. Normal NO bioactivity helps to inhibit the expression of adhesive molecules. It can be concluded that both vitamin C supplementation and low intensity exercise training are recommended for diabetic patients in order to prevent diabetic cardiovascular complications.

In summary, we suggest that both regular low-intensity exercise training and vitamin C will be beneficial in preventing diabetes-induced endothelial dysfunction by *different underlying mechanisms* as shown in **Fig. 2**. The proposed mechanisms are that low-intensity exercise training can decrease ROS through the prevention of diabetes-induced hyper-triglyceridemia. Vitamin C can scavenge ROS, superoxide (O^{-2}), and hydrogen peroxide (H_2O_2), and can increase or preserve nitric oxide (NO) bioavailability. This benefit response mediated via NO bioavailability can be supported by our previous study where vitamin C could preserve eNOS expression in myocardium of diabetic rats [33]. Normally NO helps to inhibit the expression of adhesive molecules, thus, preventing leukocyte-endothelial cell interactions. It can be concluded that both vitamin C supplementation and low intensity exercise training are recommended for diabetic patients in order to prevent diabetic cardiovascular complications.

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