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Full Length Research Paper

Effects of curcumin and tetrahydrocurcumin on diabetes induced endothelial dysfunction

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Diabetes-induced endothelial dysfunction has been well recognized for its underlining causes of oxidative stress. Therefore, several studies have worked on testing active antioxidant agents such as vitamin C and E against diabetes induced oxidative stress to preserve endothelial cells. In the present study, we assessed the effects of plant-derived antioxidant, curcumin and its analog, tetrahydrocurcumin (THC) and also its preventive qualities. Using an animal model, assessment for endothelial-dependent vasodilatation and the behavior of leukocytes were accomplished by using streptozotocin-induced diabetic rats and its mesenteric microcirculation parameters. The results indicated that both antioxidants, curcumin and THC, could significantly inhibit those abnormalities typically seen in endothelial dysfunctions ($P < 0.05$) in relation to their hypoglycemic and hypolipidemic properties. Unfortunately, curcumin has a poor absorption quality and as a result of this, a higher dose is needed to prevent abnormal endothelial functions. On the other hand, THC was found to be a more potent antioxidant and good therapeutic agent for diabetic patients in preventing diabetes vascular abnormalities.

Key words: Curcumin, tetrahydrocurcumin, endothelial dysfunction, diabetes.

INTRODUCTION

In diabetes mellitus, the endothelial dysfunction is characterized by the impairment of vasorelaxation and the over expression of adhesive molecules (Durante et al., 1988; Singhania, et al., 2008). This has been widely documented as an underlying cause for both diabetic macro and micro angiopathy (Amos et al., 1997; King et al., 1998). It has been pointed out that an increase in reactive oxygen species (ROS), subsequently produced by the polyol pathway, nonenzymatic glycation, redox potential alterations and stimulation of the diacylglycerol (DAG)-protein-kinase C (PKC) pathway, all contribute significantly to induce diabetic endothelial dysfunction (Van der Jagt et al., 2001; Bunnag, 2006). The potential contribution of the increased ROS to the development of endothelial dysfunction in diabetes has received a lot of considerable interest, since it interferes with the production of nitric oxide (NO), a key factor in multiple processes of the vascular functional homeostasis. As a result of this, several studies have investigated the roles of antioxidant agents and its benefits as a vasculoprotective agent in diabetic patients (Ting et al., 1996; Park et al., 2000; Jariyapongskul et al, 2002).

For example, recently, there has been an increased clinical interest in curcumin (diferuloylmethane), an extract of turmeric root and its potent antioxidant property in reducing hyperlipidemia and its anti-inflammatory quality (Paoletti 1962; Patil and Srinivasan, 1971; Soni and Kuttan, 1992; Soudamini et al., 1992). Aside from that, curcumin has several other therapeutic attributes such as

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Abbreviations: STZ; streptozotocin-induced diabetes rat, **STZ-vit C**; streptozotocin-induced diabetes rat treated with vitamin C (1 g/L mixed drinking water, freely assess), **STZ-cur**; streptozotocin-induced diabetes rat treated with curcumin (300 mg/Kg, oral feeding), **STZ –THC**; streptozotocin-induced diabetes rat treated with tetrahydrocurcumin (100 mg/Kg, oral feeding

preventing biliary disorders, anorexia, coughs, diabetes, hepatic disorders, rheumatism, sinusitis, cancer and Alzheimer disease (Araujo CAC and Leon LL., 2001; Aggarwal et al., 2003). As of note, especially in diabetes, it has been shown that curcumin has the ability to delay the development of cataracts (Suryanarayana et al., 2005), ameliorate renal lesions (Babu and Srinivasan, 1998) and reduce cross-linking of collagens (Sajithlal et al., 1998) in a streptozotocin-treated diabetic animal model.

These antidiabetic properties seen in curcumin make it an attractive substance to further investigate to see if there are additional benefits not reported in literature. Interestingly, it has also been reported that tetrahydrocurcumin (THC), an analog of curcumin, is a more potent antioxidant than curcumin (Okada et al 2001; Pari and Murugan 2004). Collectively, with this information (Murugan et al., 2006), we decided to evaluate the effects of curcumin and its analog, THC, to determine whether they can protect endothelial cells against diabetes induced oxidative stress.

MATERIALS AND METHODS

Experimental protocol

30 male Wistar Furth rats (200 - 250 g BW) were obtained from the national laboratory animal center, Salaya campus, Thailand. Experiments were conducted in accordance with the guidelines for animal experimentation of the national research council of Thailand and approved by the ethics committee, faculty of pharmacy, Chulalongkorn University. The rats were randomly divided into non-diabetic (control; n = 6) and diabetic groups (n = 24). Diabetes was induced by a single intravenous injection of streptozotocin (STZ; sigma chemical Co., St. Louis, USA, 55 mg/kg BW). On day 0, STZ was made fresh by dissolving it in citrate buffer with a pH of 4.5 (Sigma chemical Co., St. Louis, USA) and was immediately injected into the experimental group. In parallel, on day 0, the control group (Con) was injected with 0.9% sterile saline solution. Diabetes was defined as having a glucose concentration of more than 200 mg/dl and this was verified by using a glucometer (Advance glucometer, Boehringer Mannheim, Germany) 48 h after the rats were injected with STZ (Patumraj et al., 2006).

The diabetic rats were further divided into 4 subgroups: STZ rats (STZ; n = 6), STZ-treated with vitamin c supplementation (L-ascorbic acid, 99% sigma chemical Co., 1 g/L mixed in drinking water [17 - 18]) (STZ-vitC; n = 6), STZ-treated with curcumin ($C_{21}H_{20}O_6$; 1,7-bis (4-hydroxyl-3-methoxyphenyl) 1E,6E-heptadiene-3,5-dione; Cayman Chemical Co., USA) (300 mg/Kg.BW, daily oral feedings) (STZ-cur; n = 6) and STZ-treated with tetra-hydrocurcumin ($C_{21}H_{24}O_6$, prepared by department Of chemistry, faculty of science, Ramkamhang university) (100 mg/kg BW, daily oral feedings) (STZ-THC; n = 6). Daily oral intake of curcumin and THC were started on day 10 as well as vitamin C (Pargalava et al., 2004; Halim et al., 2002; Leelavinothan et al., 2007; Amatyakul et al., 2003). Curcumin and THC were dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma, USA).

On week 8, all rats were anesthetized intraperitoneally (i.p.) with 60 mg/kg of pentobarbital sodium. A jugular vein and carotid artery were canulated for injection with fluorescence tracers and blood pressure readings were recorded. The rats were allowed to stabilize for 20 - 30 min following surgery before placing the animal on the stage of a fluorescence microscope. Mesenteric microcirculation was observed *in vivo* after iris blood perfusion flow was measured.

Studies of mesenteric arteriolar response to vasoactive agents

To observe the mesenteric microcirculation, the abdomen was opened and the small intestine was displaced to expose a segment of the mesentery. A well-vascularized mesenteric window was selected and spreaded out flat over a small plexiglass platform. The mesentery was kept warm and moist by continuous superfusion with Krebs-Ringer-bicarbonate-buffered solution at 37°C and was observed under a fluorescence videomicroscope (Nikon, Tokyo, Japan) with a x20 objective lens. Microvessels were labeled with 5% FITC-dextran 250 (Sigma, St. Louis, USA) which was injected into the jugular vein (0.2 ml) [17 - 18]. After preconstriction arterioles with norepinephrine (NE; 10^{-5} M, sigma chemical Co., St. Louis, MO, USA), the vasodilatation of arterioles with acetylcholine (Ach; 10^{-5} M; sigma chemical Co., St. Louis, MO, USA) and sodium nitroprusside (SNP; 10^{-5} M; Sigma chemical Co., St. Louis, MO, USA) was conducted by using intravital fluorescent videomicroscope. The changes of the mesenteric arteriolar diameters, before and after each vasodilator was administered, were recorded real time throughout the experiment with a black and white video monitor (Sony, GM-1411 QM) and a silicon intensified target television camera (Nikon-SIT 68, Tokyo, Japan) mounted on a fluorescence microscopy using a x20 objective lens and a x10 eyepiece (CFI Plan Fluor). Video images of microvessels were stored on videotape (Sony, DX-E 120, Tokyo, Japan) connected to a video timer. Diameter of mesenteric microvessel images (20 - 40 *u*m in diameter) was measured using the software Global lab image II, (Data Translation, USA). The arteriolar diameter was calculated by averaging 3 measurements obtained from 3 different video frames using the same reference point as a marker for measuring each vessel in each frame. Arteriolar diameters were measured 5 min after Ach or SNP was administered. Vasodilatation responses were expressed as the % of maximal relaxation after preconstriction norepinephrine (NE; 10^{-5} M).

Evaluation of leukocyte adhesion

In order to assess the number of leukocyte adherence,

Table 1. These are the metabolic parameters for the controls (Con), streptozotocin (STZ), STZ-treated with vitamin C (STZ-vitC), STZ-treated with curcumin (STZ-cur) and STZ-treated with tetrahydrocurcumin (STZ-THC) groups at 8 weeks after STZ-injection (Mean ± SEM).

Group	Blood glucose (mg/dl)	HbA1c (%)	Malondialdehyde in liver In mole/100 mg wet wt)
CON	83.13 ± 7.70 (n = 6)	4.15 ± 0.33(n = 6)	58.36 ± 27.77 (n = 6)
STZ	523.00 ± 16.49**(n = 7)	10.86 ± 0.37*(n = 7)	157.26 ± 14.62** (n = 7)
STZ-vitC	380.13 ± 18.61$^{\#}$ (n = 6)	9.68 ± 0.03 $^{\#}$ (n = 6)	67.15 ± 5.90$^{\#}$ (n = 6)
STZ-cur	358.33 ± 33.05 $^{\#}$ (n = 8)	9.13 ± 0.28 $^{\#}$ (n = 8)	96.50± 6.80 $^{\#}$ (n = 8)
STZ-THC	386.20 ± 23.98$^{\#}$ (n = 6)	9.22 ± 1.08$^{\#}$ (n = 6)	100.25 ± 4.34$^{\#}$ (n = 6)

*P < 0.05; Significantly different compared to Con group
**P < 0.001; Significantly different compared to Con group
$^{\#}$P < 0.05; Significantly different compared to STZ group
^{ns} not significantly different compared to STZ group (P < 0.05).

the mesenteric tissues were prepared similarly to that described earlier. Instead of using FITC-dextran 250, rhodamine 6G (Sigma, St. Louis, USA) was used for labeling the leukocytes (Aggarwal et al., 2003; Jariyapongskul et al., 2003). 0.3 ml rhodamine 6G (conc. 0.3 mg/ml; Sigma, St. Louis, USA), a total of 0.09 mg rhodamine per animal, was injected into the rat's jugular vein. Based on the rhodamine video image of each experiment, leukocytes were regarded as adherent when the cells remained stationary for more than 30 s. For each rat, 2 or 3 single vessels were selected from the iris microvascular network to assess the number of leukocytes adhering to the vessels. The number of leukocytes adhering to the venules' endothelium (20 -50 *u*m in diameter) was counted by using the software Global lab image II.

Iris blood perfusion

The iris blood flow perfusion was measured by using a laser Doppler flowmeter (model ALF 21, Japan) with a fiber optic needle probe (wavelength 780 nm; 1 mm diameter). The needle probe was fixed 1 mm above the iris and perpendicular to the tissue. The blood perfusion was measured at 8 different locations within the iris tissue at a time and the averaged value was used for each rat.

Measurement of metabolic parameters

At the end of each experiment, blood sample of each rat was collected. Blood glucose (BG), lipid profiles and glycosylated hemoglobin (HbA_{1C}) were measured from the blood samples using the enzymatic method by RIA laboratory Co, Ltd. (Bangkok, Thailand).

After the intravital microscopic observation, the liver of each animal was perfused with cold 0.1 M PBS and then immediately excised and immersed in an ice-cold 0.1 M PBS. Fat and fibrous tissues were removed before weighing each liver. All livers were collected to assess malondialdehyde (MDA) levels using thiobarbituric acid reaction described by Ohgawa et al. (1979).

Data analysis

All data were presented as mean ± SEM (standard errors of mean). For comparison among groups of animals, 1 way analysis of variance (one-way ANOVA) was used. Tukey's test was used to compare the difference between the means of diabetic rats and the controls and between diabetic-untreated and diabetic-treated animals. If the statistical probability (p - value) was less than or equal to 0.05, the difference was considered to be statistically significant.

RESULTS

Changes in blood glucose, HbA_{1C} and MDA

Table 1 shows the levels of blood glucose and HbA_{1C} measured for each group. The blood glucose and HbA_{1C} of all diabetic groups (STZ, STZ-vitC, STZ-cur and STZ-THC) were significantly higher than those of the Con (P < 0.001). Among STZ, STZ-vitC, STZ-cur and STZ-THC, there was a significant difference in blood glucose and HbA_{1C}. As for liver-malondialdehyde (MDA) level, an indicator of oxidative stress, only STZ group had a significant MDA level at week 8 which was almost 2-fold higher than those of the Con (P < 0.001). On the other hand, the MDA levels of 3 treated groups, STZ-vitC, STZ-cur and STZ-THC, were significantly lower than those of age-matched untreated diabetic rats (P < 0.05).

Changes in lipid profile

Table 2 shows the level of lipids (serum cholesterol, TG, HDL-c and LDL-c) measured for each group. There was a significant increase in cholesterol, TG and LDL-c in untreated STZ group compared to their age-matched controls. Notably, cholesterol, TG and LDL-c in STZ-cur and STZ-THC groups were significantly lower compared to the untreated STZ group (P < 0.05), whereas vitamin C treated group did not seem to have any changes in diabetic dyslipidemia. Thus, curcumin and THC appear to

Table 2. These are the lipid profiles for the Controls, streptozotocin (STZ), STZ-treated with vitamin C (STZ-vit C), STZ-treated with curcumin (STZ-cur), STZ-treated with tetrahydrocurcumin (STZ-THC) groups at 8 weeks after STZ-injection. Results are shown as Mean ± SEM.

Group	Cholesterol(mg/ml)	TG (mg/ml)	LDL-c(mg/ml)	HDL-c(mg/ml)
CON	31.33 ± 3.21	51.17 ± 1.45	3 ± 0.45	45.50 ± 2.43
STZ	76.67 ± 7.68**	73.67 ± 2.08**	12.88 ± 0.86*	30.50 ± 3.01*
STZ-vit C	91.80 ± 3.59ns	121.00 ± 2.08#	15.33 ± 0.42ns	54.80 ± 2.44#
STZ-cur	43.00 ± 1.53*,#	34.33 ± 1.67#	6.33 ± 0.33*	38.17 ± 1.40ns
STC-THC	51.00 ± 5.54*,#	43.20 ± 10.80*,#	7.67 ± 1.48*,#	41.33 ± 6.52#

*P<0.05; Significantly different compared to Con group
**P<0.001; Significantly different compared to Con group
# P<0.05; Significantly different compared to STZ group
ns not significantly different compared to STZ group (P < 0.05)

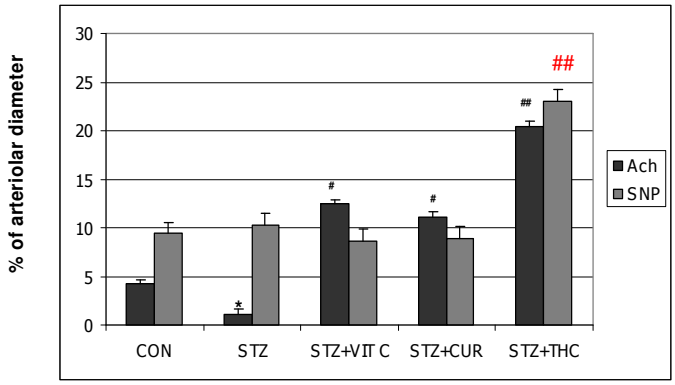

Figure 1. Iris blood flow perfusion measurements for the Controls (n = 6), STZ (n = 6), STZ-vit C (n = 6), STZ-cur (n = 6), and STZ – THC (n = 6). Results are shown in Means ± SEM.
Significantly different compared to STZ group (P < 0.05); ns Not significant compared to STZ group (P < 0.05); **Significantly different compared to control (P < 0.01).

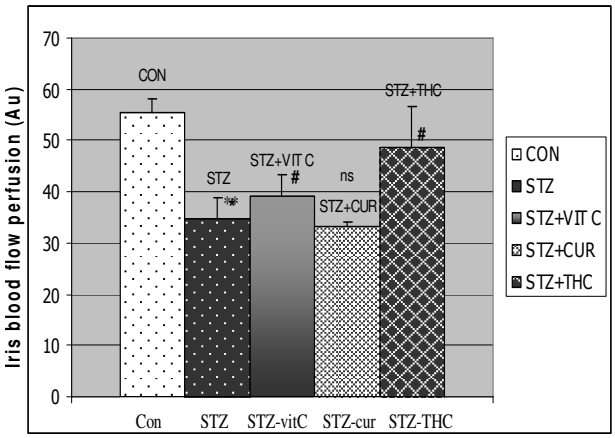

Figure 2. Measurements of endothelial-dependent vasodilatation for the controls (n = 6), STZ (n = 6), STZ-cur (n = 6), STZ-tetra (n = 6). Results are shown as Means ± SEM.
#Significantly different compared to STZ (P < 0.05).); ## Significantly different compared to STZ (P < 0.001);
*Significantly different compared to control (P < 0.05).

have hypoglycemic and antilipidemic effects in STZ induced diabetic rats.

Changes in iris blood perfusion

Figure 1 shows iris blood flow perfusion measured in all diabetic groups (Con = 55.35 + 2.96, STZ = 34.75 + 4.26, STZ-cur = 33.11 + 4.34, STZ-THC = 48.77 + 8.44). It was shown that iris blood flow perfusion in diabetic rats was significantly lower than the controls. However, it is possible that THC is increased in tissue perfusion due to diabetes (P < 0.05). Curcumin could not significantly prevent this change of iris blood flow perfusion.

Endothelium-dependent relaxation

Endothelium-dependent relaxation was determined by measuring the mesenteric arteriolar responses to topical application of Ach (10^{-5} M). The changes of arteriolar diameter in each group were reported in % of change from baseline diameter.

In Figure 2, the results showed that there was a significant decrease in arteriolar responses to Ach and not to SNP in diabetic rats (STZ; Ach = 1.16 + 0.35, SNP = 10.32 + 2.18) when compared to the controls (control; Ach = 4.18 + 0.51, SNP = 9.39 + 1.21) (P < 0.05). Interestingly, both curcumin and THC could significantly increase arteriolar responses to Ach caused by diabetes (P < 0.001). It is worthy to mention that THC appears to have a more potent antioxidant effect in the preservation of the endothelium than vitamin C and curcumin. Ach-induced vasodilatation was approximately 1.67 times higher in the THC group (20.41 + 4.49) when compared to vitamin C (12.42 + 1.11) or curcumin (11.07 + 1.17) treated groups. Moreover, the results also showed that THC (23.09 + 4.65) was able to increased SNP-induced vasodilatation up to 2.3 times higher than STZ (10.32 ± 2.18), while curcumin appears no difference.

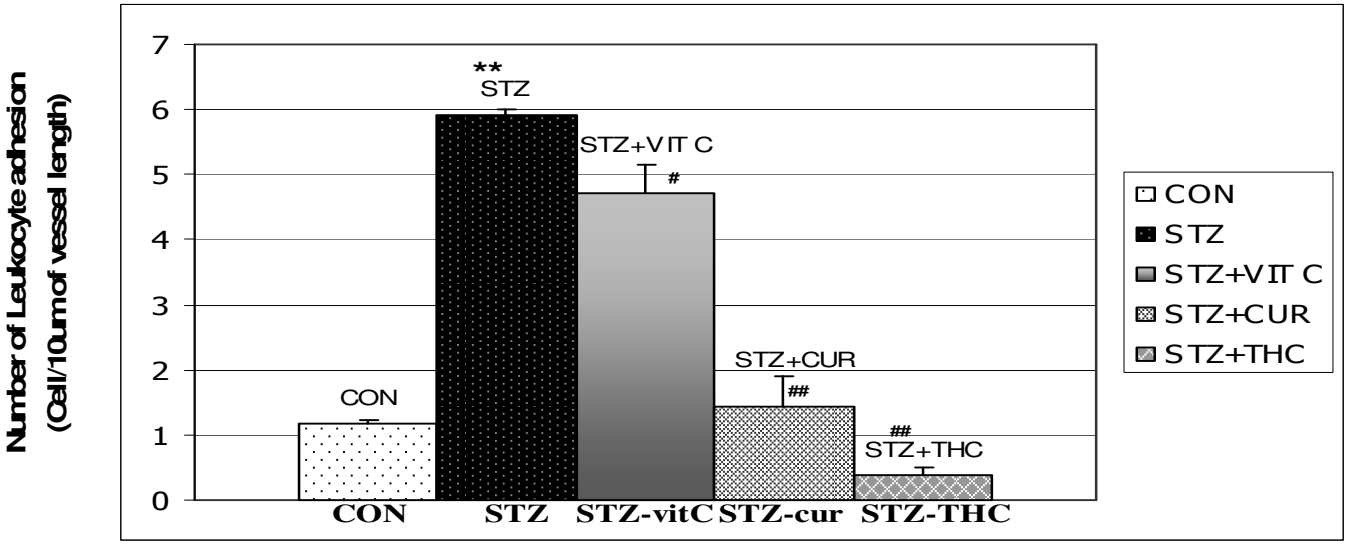

Figure 3. Number of leukocytes adhering to the endothelium calculated from mesenteric video images obtained from each group: Controls (n = 6), STZ (n = 7), STZ-vit C (n = 6), STZ-cur (n = 8) and STZ –THC (n = 6). Results are shown as Means ± SEM. ** Significantly different compared to the Controls (P<0.05); # Significantly different compared to STZ group (P<0.05); ##.

Leukocyte adhesion to endothelium

Based on the fluorescence video image, the number of leukocytes adhering to mesenteric venules is presented in Figure 3. At week 8, leukocyte adherence was found to be significantly greater in STZ untreated rats compared to the controls ($P < 0.001$), STZ-vitC, STZ-cur and STZ-THC ($P < 0.05$).

DISCUSSION

Our results demonstrated that curcumin and THC have potential effects in preventing diabetes-induced endothelial dysfunction which are characterized by the impairment of Ach-activated vasorelaxation and the increased leukocyte-endothelial cell interaction. Interestingly, the results showed that THC, not curcumin, could significantly increased SNP-induced vasodilatation in diabetes. However, more experimental data are required to explain how THC can enhance smooth muscle cell relaxation when activated by NO donor.

These findings seem to be the first *in vivo* evidence for demonstrating the potential of THC in protecting endothelial cells against leukocytes adhesion in diabetic rats. In particular, its beneficial effects appear to be comparable with vitamin C, the most common antioxidant agent used in cardiovascular protection. In our study, we also confirmed curcumin and THC to have antioxidant, hypoglycemic and lipid lowering properties. As of note, vitamin C has shown no effects on diabetic hyperlipidemia. Other studies reported curcumin's hypolipidemic effect to be dependent upon cholesterol catabolism and cholesterol 7*a*-hydroxylase, this step converts cholesterol into bile acid which is an important pathway in the degradation of cholesterol (Soni and Kuttan, 1992; Leelavinothan and Pidaran, 2007; Soudamini et al., 1992). It is unclear whether the hypolipidemic effect of THC is similar to that of curcumin since both substances own alkaloid components. Nevertheless, this warrants for additional study. Meanwhile, Halim et al. (2002) suggested that the hypolipidemic effect of curcumin might be dependent on its alkaloid component.

In another animal studies, (Jariyapongskul et al., 2008; Leelavinothan and Pidaran, 2007), they demonstrated the effects of THC in diabetic rats (THC, 80 mg/kg body weight) to have an antidiabetic effect in significantly reducing blood glucose and increasing insulin in plasma, respectively. The present study has also supported the hypoglycemic effects of curcumin and

THC. The hypoglycemic effect was also observed in STZ-vit C group which was similar to the results reported from our previous study where we had used STZ-rats aged 12 and 24 weeks (Jariyapongskul et al., 2002; Patumraj et al., 2006). It has been suggested that the antioxidant activity of vitamin C may have been produced to protect glucose transporter 1 (GLUT-1) and therefore has a hypoglycemic effect (Kim et al., 2006). They found that high levels of glucose increased oxidative stress which induced Akt activation and downregulation of GLUT-1. It is possible to hypothesize that there are 2 mechanisms for the antioxidant's hypoglycemic effects: 1) scavenging directly for reactive oxygen species and/or 2) inhibiting Akt activation to indirectly protect GLUT-1. Based on these reports, the hypoglycemic effects of curcumin and THC may be involved and will need to be confirmed in future studies.

In our experiment, on week 8, it was clearly visible that there was a significant increase in leukocyte adhesion in

the diabetic rats compared to the controls. This enhancement in leukocyte-endothelium interaction was also observed in the diabetic rat's cerebral microcirculation (Jariyapongskul et al., 2002). Interestingly, the present study showed that vitamin C, curcumin and THC were able to reduce diabetes-induced leukocyte adhesion to the endothelium.

Unlike curcumin, there is scant data on the effects of THC in diabetes-induced leukocyte-endothelium interaction. However, it has been reported that THC is a more potent antioxidant (Osawa et al., 1995) because of its unique ability to scavenge oxygen free radicals better than other antioxidant agents and as a result, will significantly decrease oxidative stress, especially in endothelial cells.

There are several studies indicating a reduction of nitric oxide (NO) in the endothelium of diabetic vessels (Hink et al., 2001). The decrease in NO may be a major contributing factor in causing a decrease in iris blood perfusion but enhancing leukocyte adhesion. The mechanisms of NO in leukocyte regulation and recruitment remains unclear even though it has been reported that it could inhibit leukocyte adhesion (Alison et al.,1998; Nolan et al., 2008).

The increase in hyperglycemia induced oxygen-derived free radicals was believed to be a major contributor in reducing NO bioavailability in diabetes. In our study, we did not monitor the NO levels but used an alternative technique, Ach activation, to show endothelial-dependent vasodilatation. From the MDA levels (the common oxidative stress indicator), we were able to indirectly see a reducetion of oxidative stress attributed to the effects of curcumin and THC through the decreased levels of BG and hyperlipidemia. This value is inversely proportional to the increase in NO bioavailability.

Conclusion

In conclusion, our findings indicate curcumin and THC are effective in protecting the function of endothelial cells against diabetes-induced dysfunction. Through our study, we were able to show curcumin and THC function as antioxidants with hypoglycemic and hypolipidemic actions. Therefore, our findings suggest that in the near future, vitamin C, curcumin and THC may be used as therapeutic agents for protecting diabetes-induced endothelial cell dysfunction which is a major underlining cause for diabetic cardiovascular complications.

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