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Polyphenolics-Rich *Psidium guajava* Budding Leaf Extract Can Reverse Diabetes-Induced Functional Impairment of Cavernosal Smooth Muscle Relaxation in Rats

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Abstract: Diabetes Mellitus (DM) related Advanced Glycation End products (AGEs) are considered to induce functional impairment of cavernosal smooth muscle relaxation and cause Erectile Dysfunction (ED). We used rats to examine the *in vitro* effects of *Psidium guajava* L. (Myrtaceae) budding leaf extract (PE) on pharmacological relaxation of corpus cavernosum smooth muscle strips obtained from diabetic rats. After 8 weeks, the mean glycosylated haemoglobin (HbA1c), serum cholesterol and triglyceride concentrations were significantly higher in the non-PE diabetic than in the age-matched control animals. Interestingly, in diabetic animals fed PE, serum cholesterol and triglyceride levels were significantly lower than in their peers given a standard diet. The administration of PE to diabetic animals for 8 weeks reversed the expected impaired relaxation response and nitric oxide production in cavernosal smooth muscle exposed to acetylcholine or electrical field stimulation. The administration of PE to rats with 8 weeks of uncontrolled diabetes reverses DM-induced harmful effects on vascular smooth muscle.

Key words: Diabetes mellitus, erectile dysfunction, *Psidium guajava*, corpus cavernosum

INTRODUCTION

Erectile Dysfunction (ED) is prevalent in men with Diabetes Mellitus (DM) (McCulloch *et al*., 1980). The pathogenesis in cavernosal smooth muscle from chronic hyperglycemia-related Advanced Glycation End product (AGE) formation causes functional impairment of smooth muscle relaxation (Cartledge *et al*., 2001) and leads to diabetes-related erectile dysfunction (Seftel *et al*., 1997).

The relevant phenomenological changes associated with endothelial cells include slowing of the cell growth rate (Rojas *et al*., 2003), significant extension of the cell cycle (Lorenzi *et al*., 1987), increased consumption of antioxidative substances (Curcio and Ceriello, 1992) and more severe cell apoptosis (Wu *et al*., 1999; Du *et al*., 1999). Major complications in endothelial cells in chronic diabetes include atherosclerosis and neurodegeneration. Hence current major pharmacological research is concerned with preventing these detrimental effects (Suh *et al*., 2003).

The non-enzymatic condensation reaction between reducing sugars (such as glucose) and amino acid chains in proteins (also called the Maillard reaction or glycation) has been shown to play an important role in the development of chronic complications in Diabetes Mellitus (DM) (Aronson and Rayfield, 2002). The appearance of intermediates leading to the formation of Amadori compounds occur in the early stage of glycation, while in the late stage, Advanced Glycation End products (AGEs) are irreversibly formed after a complex cascade of repeated reactions such as dehydration, condensation, fragmentation, oxidation and cyclization (Kikuchi *et al*., 2003).

So called Complementary Alternative Medicines (CAM) have attracted tremendous attention from physicians of conventional western medicine

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(Cassileth and Deng, 2004). Many of these in fact have been observed to be more effective and versatile and less toxic than conventional clinical treatments. More importantly, a safer prognosis than with other therapies has been reported (Furusawa and Furusawa, 1989, 1990). Compounds with antioxidant biological activities in dietary supplements have gained considerable interest (Joshi *et al*., 2001; Narayanan *et al*., 2005).

Psidium guajava, commonly known as guava, belongs to the family Myrtaceae and is an important tropical fruit in Taiwan. Guava leaves are frequently utilized as a folk medicine and as an astringent hemostatic, as well as a folk therapeutic in the treatment of diabetes and enteritis (Ojewole, 2005; Iwu, 1993). Some investigators suggested that the active components in guava fruits are ursolic acid, oleanolic acid, arjunolic acid and glucuronic acid, while β-sitosterol glucoside and brahmic acid are present in guava leaves (Ojewole, 2005). In addition, we have successively identified seven bioactive polyphenolic compounds from guava budding leaves, including catechin, epicatechin, gallic acid, quercetin, rutin, naringenin and kaempferol (Hsieh *et al*., 2007). In the recent study (Hsieh *et al*., 2005), we showed that the polyphenolic content of *Psidium guajava* budding leaf extract (PE) has excellent antiglycation effects and free radical scavenging ability.

Despite these promising findings, the role of PE has not been studied in diabetic ED in which AGEs are considered to induce the functional impairment of cavernosal smooth muscle relaxation. Therefore, we used rats to examine the *in vitro* effects of PE in preventing DM-induced ED, since rat cavernosal tissue is a good model of cavernosal smooth muscle function (Cartledge *et al*., 2001).

MATERIALS AND METHODS

Extraction of PE: Two hundred grams of *Psidium guajava* budding leaf was boiled in 200 mL of water for 30 min, filtered through Whatman No. 2 filter paper and lyophilized into a pulverized form. The yield was 9.10 g.

Animal model establishment: The studies were carried out in accordance with the principles and procedures of the Animal Ethics Committee of Hungkuang University. All animals were male, sexually mature Sprague-Dawley rats. Tissue from age-matched control group animals (n = 8) was harvested from 24-weeks-old animals; the other group of animals (n = 8) were 16 weeks old at the induction of diabetes, which was maintained until 24 weeks old without treatment. Diabetes was induced by an intraperitoneal injection with streptozotocin (60 mg kg^{-1}) mixed with 0.05 mol L^{-1} sodium citrate and sterile water for injection. Blood was taken from a tail stab to measure glucose using reagent strips (ACCU-CHEK Advantage II test strips, Roche Diagnostics, Mannheim, Germany). Diabetic animals were weighed at the time of induction of diabetes and then daily thereafter. The group of age-matched control animals had blood drawn for baseline glucose measurement and were weighed every 7 days. Two further groups of age-matched control group (n = 8) and diabetic rats (n = 8) were given free access to a PE-treated standard rat diet to provide an oral dose of 1.5 mg/g/day, which was started at the time of induction of diabetes and continued until death.

After 8 weeks, the 2 groups of animals with diabetes were killed by cervical dislocation and their penises rapidly dissected and placed in chilled Krebs solution (Cartledge *et al*., 2000). Blood was drawn from a tail stab for blood glucose estimation and from the left atrium for the measurement of serum electrolytes, lipids and glycosylated Haemoglobin (HbA1c). The other 2 groups of age-matched control animals were killed and treated in the same way. At the time of death, penile tissue from all four groups was prepared and mounted in an organ bath.

Experimental protocols: In the beginning of the experiments, the penile tissues were contacted with 30 μM phenylephrine to obtain a maximal response. Once the maximal response had been obtained, the penile tissues were washed every 20 min with Krebs solution until the tension returned to the basal level. The contractions were recorded after adding 30 μM phenylephrine and then responses to incremental doses of acetylcholine, or Electrical Field Stimulation (EFS) instead of acetylcholine in the presence of guanethidine (70 μM) and atropine (3 μM) were also recorded. Frequency-response curves were obtained by stimulating the tissue strips with a train of square-wave pulses (2, 5, 10, 20 and 50 Hz) (band width 0.5 ms, intensity 20 V). A rest interval of 12 min was given between two stimulations. Preliminary experiments showed the reproducibility of the responses at all the frequencies tested in tissue strips from all four experimental groups of rats.

Measurement of NO release: The Nitric Oxide (NO) levels in the corpous cavernosal tissue strips were measured by determining the concentration of nitrite using High Performance Liquid Chromatography (HPLC) based on the Griess method (Rahbar, 2005). Dialysate (20 μL), which was obtained from microdialysis of the corpous cavernosal strips precontracted with 30 μM

phenylephrine (Kuwahara *et al*., 2003) followed by incremental doses of acetylcholine or Electrical Field Stimulation (EFS) with or without previous 100 μM N^G-Nitroarginine Methyl Ester (L-NAME) exposure, was injected into the NO detector-HPLC system (ENO-20; Eicom, Address). The levels of nitrite were determined by measuring the absorbance of the colour product at 540 nm by a flow-through spectrophotometer (NOD-10; Eicom).

Tissue preparation: Penile tissue from all four groups of animals was treated according to procedures in the report of Cartledge *et al*. (2000). Briefly, with the aid of a dissecting microscope, the corpus cavernosa was cleaned of overlying fascia and muscle and separated from the thick, medial, tunica albuginea, which adjoins the paired corpora cavernosae. All dissecting procedures were done with extreme care to protect the corpora cavernosa from inadvertent damage. The cavernosal strips (3×3×12 mm) obtained were mounted in a 20 mL organ bath perfused with Krebs solution gassed with 95% O_2/5% CO_2 and maintained at 37°C for the measurement of isometric tension. The Krebs solution was exchanged every 15 min from a stock reservoir gassed with 95% O_2/5% CO_2 and maintained at 37°C.

Statistical analysis: Relaxation responses of tissue strips to acetylcholine or EFS were represented as a percentage of the maximal contraction achieved for that strip in response to 30 μM phenylephrine. Student's t-test was used to compare a single variable and two-way ANOVA to compare the responses of two different tissue samples, or of a single tissue sample under different conditions, to a range of doses of acetylcholine or EFS. A significance level of $p<0.05$ was applied for all analyses. ANOVA generated an overall response for the range of variables under examination; this value was given as the mean overall relaxation response and was used in discussing the effects of tissue strips under different conditions.

RESULTS

Body weights and blood parameters: The mean weight of the experimental animals at the induction of diabetes was 403.6±3.2 g. Diabetes was induced in 2 groups of animals (n = 16), one group of eight with uncontrolled diabetes that received a standard diet and water and the other group of eight that were fed PE from the time of induction of diabetes. All animals survived the experimental period. All diabetic animals had an elevated HbA1c after 8 weeks, which was consistent with a prolonged period of hyperglycaemia. The essential characteristics of the controlled and PE-treated diabetic animals are given in Table 1. After 8 weeks of uncontrolled diabetes there was a markedly significant increase in serum cholesterol and triglyceride concentrations over age-matched control animals given an identical diet (Table 1). In diabetic animals fed PE, serum cholesterol and triglyceride levels were significantly lower than in their peers on a standard diet.

Table 1: The basic characteristics or the rats, the baselinevalues of the control (24 weeks) and diabetic animal groups with or without PE

Characteristics	Control group	Diabetic group	24 weeks control +PE	Diabetic group +PE
Weight				
Animal (g)	523.6±15.3	347.2±16.1*	556.4±13.8	511.5±8.3#
Tissue strip (mg)	72.3±2.1	63.5±1.4*	71.6±0.5	70.8±0.8#
Serum data				
Sodium (mmol L^{-1})	134±1	129±0.7*	139±1	130±0.8
Potassium (mmol L^{-1})	10.1±0.4	10.5±0.5	10±0.3	9.8±0.5
Creatinine (μmol L^{-1})	51.5±0.4	63.1±2.3*	50.8±1.6	65.8±1.5
Cholesterol (mmol L^{-1})	1.6±0.04	3.9±0.3*	1.7±0.03	2.3±0.1#
Triglyceride (mmol L^{-1})	2.5±0.02	11.0±1.2*	2.3±0.2	7.5±1.3#
HbA_{1C} (%)	4.51±0.05	12.7±0.3*	4.38±0.7	11.90.4
Glucose (mmol L^{-1})	8.44±0.5	32.5±1.2*	9.23±0.6	33.4±0.7

Significant difference between *diabetic and age-matched control and #diabetic animals and diabetic animals fed PE

***In vitro* studies:** The mean weight of the cavernosal tissue strips harvested from non-PE-treated diabetic animals was significantly less than that from both PE-treated diabetic and age-matched control animals (Table 1). The mean contractile force of the cavernosal tissue strips harvested from PE-treated diabetic animals in response to 30 μM phenylephrine, at 346.3±15.6 mg, was not significantly different than that generated in strips from diabetic animals given a routine diet, at 377.5±19.7 mg. PE-treated control animals yielded corpus cavernosal strips which generated a contractile force of 428.5±48.4 mg in response to phenylephrine, which was not significantly different from age-matched control animals fed a standard diet, at 445.1±32.6 mg. There was no significant difference recorded when the cavernosal tissue strips from control animals fed PE and those given a standard diet were precontracted with 30 μM phenylephrine followed by incremental doses of acetylcholine (Fig. 1). The mean overall relaxation response to a range of doses of acetylcholine in these two groups was 41 and 41.2%, respectively. Rats with 8 weeks of uncontrolled diabetes had a lower relaxation response after acetylcholine than those with PE-treated diabetes ($p<0.05$), with an overall reduction in relaxation of 23 and 39.8%, respectively (Fig. 1). There was no significant difference in the relaxation responses recorded in cavernosal strips derived from diabetic animals fed PE and control age-matched animals given either a standard diet or PE (Fig. 1). PE had no effect on the responses to EFS in cavernosal tissue derived from control animals (Fig. 2). Diabetes caused a significant overall impairment in the

Table 2: Measurement of NO release in experimental groups (pmol g^{-1} weight)

NO release	Control group	Diabetic group	24 weeks control+PE	Diabetic group+PE
Basal value	672.5±64.3	449.6±58.8[a]	685.4±71.5	613.8±52.5[d]
Value during acetylcholine relaxation	1439.7±153.9	1053.4±144.6[b]	1525.1±123.5	1388.9±141.6[e]
Value during acetylcholine relaxation with L-NAME	709.6±54.3	516.8±63.8	725.4±60.6	638.3±72.4
Value during EFS	1543.7±166.3	957.6±138.5[c]	1674.8±188.9	1395.4±157.5[f]
Value during EFS with L-NAME	785.6±61.7	521.3±75.5	814.5±67.4	677.5±72.6

Data are shown as mean±SD of 8 determinations in each group; [a]Significantly different of basal value from diabetic group and control group ($p<0.05$); [b]Significantly different of value during acetylcholine relaxation from diabetic group and control group ($p<0.05$); [c]Significantly different of value during EFS from diabetic group and control group ($p<0.05$); [d]Significantly different of basal value from diabetic group and diabetic group feed with PE ($p<0.05$); [e]Significantly different of value during acetylcholine relaxation from diabetic group and diabetic group feed with PE ($p<0.05$). [f]Significantly different of value during EFS from diabetic group and diabetic group feed with PE ($p<0.05$)

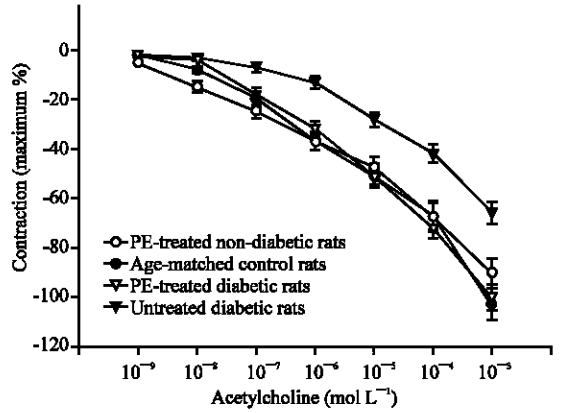

Fig. 1: The effects of PE on non-diabetic and diabetic rat cavernosal tissue responses to acetylcholine. There was no significant difference in the response of tissue from PE-treated non-diabetic rats and age matched control rats to acetylcholine. The treatment of diabetic animals with 1.5 mg/g/day PE reversed the impaired relaxation response in tissue strips exposed to acetylcholine. The relaxation response of corpus cavernosal tissue strips from PE-treated diabetic rats was significantly greater than that seen in tissue from untreated diabetic rats ($p<0.05$)

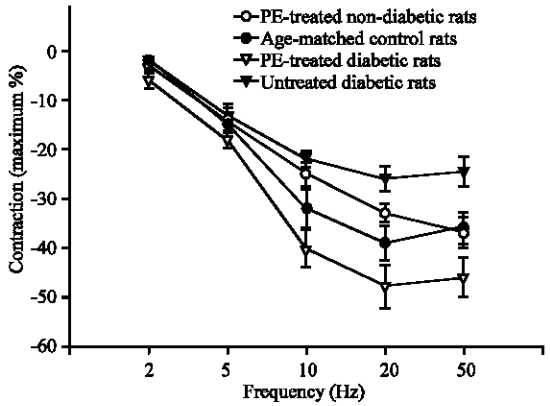

Fig. 2: The effect of PE on non-diabetic and diabetic rat cavernosal tissue responses to EFS. There was no significant difference in the response of tissue from PE-treated non-diabetic rats and age-matched control rats to EFS. The treatment of diabetic animals with 1.5 mg/g/day PE reversed the impaired relaxation response of tissue strips exposed to EFS. The relaxation response of corpus cavernosal tissue strips from PE-treated diabetic rats was significantly greater than that seen in tissue from untreated diabetic rats ($p<0.05$)

relaxation response with EFS. PE reversed this effect; the relaxation response of cavernosal tissue derived from diabetic animals fed PE was significantly greater than that seen in tissue from diabetic animals given a standard diet (Fig. 2).

Measurement of NO release: The nitrite levels in the incubated media of the corpous cavernosal tissues precontracted with 30 μM phenylephrine followed by incremental doses of acetylcholine or EFS with or without L-NAME are shown in Table 2. The corpous cavernosal tissues from uncontrolled diabetic animals not fed PE had significantly lower nitrite levels than those in the control or PE treated diabetic animals ($p<0.05$) and the nitrite levels in the control or PE treated diabetic animals were suppressed significantly by L-NAME.

DISCUSSION

Diabetes mellitus is a group of ailments characterized by abnormal carbohydrate, lipid and protein metabolism resulting from insufficient action of insulin (Rahbar, 2005). It has been reported to result in a high incidence of micro and macrovascular complications, which are inherently and pathologically associated with hyperglycemia and its subsequent damaging reaction, glycation, i.e., nonenzymatic glycosylation depends on the linkage of reducing sugars to certain residues of amino acids (Veassara and Palace, 2002).

Hyperglycemia can accelerate LDL glycation with subsequent AGE formation (Aronson and Rayfield, 2002) and AGEs may impair relaxation of vascular smooth muscle in three ways. One way is by a decrease in compliance. Altered lipid profiles such as increased

cholesterol and triglyceride impair endothelial NO mediated smooth muscle relaxation in vascular tissues by the production of Reactive Oxygen Species (ROS) (Adams *et al*., 2000). ROS may be involved in AGE formation and vice versa. We have reported that the main phrenic compounds in PE are quercetin (12.26 mg g^{-1}), gallic acid (12.18 mg g^{-1}) and ferulic acid (9.42 mg g^{-1}) (Hsieh *et al*., 2007) and all are well-known powerful ROS scanvergers, which then inhibit AGE formation. The levels of oxidizable substrates such as Amadori adducts, reactive carbonyl and dicarbonyl compounds and polyunsaturated fatty acids are increased in the blood and various tissues in diabetes. Irreversible and reticulated Low-Density Lipoprotein (LDL) from the circulation gradually binds to AGE-modified collagen in blood vessel walls. In the majority of blood vessels, this reticular binding delays the normal outflow of LDL particles which have penetrated the vessel wall and thus enhances cholesterol deposition in the intima, followed by an accelerated development of atherosclerosis (Jakus and Rietbrock, 2004). In the present study, after 8 weeks of uncontrolled diabetes, there was were marked significant increases in serum cholesterol and triglyceride concentrations over those in age-matched control animals given an identical diet (Table 1). In diabetic animals fed PE, serum cholesterol and triglyceride levels were significantly lower than in their peers on a standard diet (Table 1). This reduction in serum cholesterol and triglyceride was reported to reverse to some extent the impairment in Nitric Oxide (NO) function (Adams *et al*., 2000; Dart and Chin-Dusting, 1999) are shown in the Table 2. PE probably brings about a reduction in serum cholesterol and triglyceride by blocking the AGE modification of macrophages, as macrophages are normally responsible for clearing these lipids from the circulation (Bierhaus *et al*., 1998).

Another way AGEs may impair relaxation of vascular smooth muscle is by quenching NO activity (Bucala *et al*., 1991). NO is liberated from the nitrergic nerve and sinusoidal endothelium and acts as a non-adrenergic, non-cholinergic neurotransmitter in cavernous smooth muscles. NO is formed from L-arginine via catalysis by NO synthase isoforms.

Acetylcholine was reported to release NO from the endothelium and endothelium-dependent NO-mediated relaxation is depressed by diabetes in multiple vascular tissues, including cavernosum (Saenz de Tejada *et al*., 1989). AGE-modified proteins are formed from the covalent reaction between free amino groups of amino acids, such as arginine, lysine and the oxo group of reducing sugars (glucose, fructose, ribose etc.). In the L-arginine-nitric oxide soluble guanylyl cyclase-cyclic guanosine monophosphate pathway, which elicits penile erection, L-arginine and the related enzymes may be the targets of AGE modification in diabetes.

The third way AGEs may impair relaxation of vascular smooth muscle is by modification of Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA). NO activation of guanylate cyclase increases the concentration of cGMP, which activates Sarco/Endoplasmic Reticulum (SER) membrane-located protein kinase G (Raeymaekers *et al*., 1988) and then phosphorylates phospholamban (Cornwell *et al*., 1991). When phospholamban is phosphorylated, SERCA activity is increased resulting in an enhanced uptake of Ca^{2+} by the SER, which results in smooth muscle relaxation (Felbel *et al*., 1988). A study has shown that AGEs are formed on intracellular SERCA during diabetes and the SERCA function of regulating Ca^{2+} translocation is modified (Bidasee *et al*., 2004).

Nutraceutics which are able to reduce glycation are urgently needed as valuable and powerful adjuvants for treatment of diabetes and its complications. Although several studies have shown that Aminoguanidine (AG), a well-known therapeutic agent, can mediate a decrease in the formation of AGEs and reverse impairment of neuronal and endothelial NO-mediated penile smooth muscle relaxation in diabetic rats (Cartledge *et al*., 2000), its high chemical reactivity and toxicity (Thornalley, 2003) are important concerns. Hence, researchers are actively pursuing the development of safer agents that are capable of efficiently inhibiting glycation and are especially focusing on natural products (Wondrak *et al*., 2002; Babaei-Jadidi *et al*., 2003). Hsieh *et al*. (2005) reported that the polyphenolic content of PE is very high, reaching a gallic equivalent of 165.61 mg g^{-1}. Both polyphenolics and flavonoids are excellent free radical scavengers as well as ferrous ions chelators (Hagerman *et al*., 1998). Quercetin *in vivo* as well as *in vitro* is a good antiglycative biochemical capable of inhibiting diabetic complications (Bae and Lee, 2004) and preventing the *in vivo* oxidative β-cell damage caused by streptozotocin (Coskun *et al*., 2005) and neuro-detrimental effects (Anjaneyulu and Chopra, 2003).

Similar effects were found for ferulic acid associated with glycation of an aspartate aminotransferase model induced by D-fructose (Bousova *et al*., 2005) and for gallate as well. Nakagawa *et al*. (2002) implicating that the prevailing wide spectrum of anti-glycative and anti apoptotic bioactivities of PE can be attributed to these major constituents. Clinically, supplements with antioxidants which inhibit AGE production have been adopted in the new strategy for delaying aging, neurodegeneration and diabetic complications. In the present study, we found that rats with diabetes had a

lower cavernosal vascular smooth muscle relaxation response than age-matched controls and PE could reverse this effect (Cartledge *et al*., 2001) by using AG to modulate AGE-modified cavernosal tissue.

CONCLUSION

We found that the administration of PE to rats with 8 weeks of uncontrolled diabetes reverses the DM-induced harmful effects on vascular smooth muscle.

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