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Full Length Research Paper

Dan-Shen-Yin has integrated protective effects in diabetic atherosclerosis rat models

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Dan-Shen-Yin (DSY) is a famous traditional Chinese formula. We determined its integrated protective effects mechanisms of DSY in diabetic atherosclerosis rat model. DSY (5 or 10 g/kg/day for 4 weeks) was orally administrated to the diabetic atherosclerosis rats, which were induced by nitric oxide inhibition (L-NAME in drinking water, 1 mg/ml) plus high-fat diet. At the end of the experiments, the serum level of malondialdehyde (MDA), total antioxidant capacity (T-AOC), superoxide dismutase (SOD) and GSH-peroxidase (GPX) activities and the serum level of glutathione(GSH) were measured; The concentrations of circulating inflammatory markers C-reactive protein (CRP), interleukin-6 (IL-6) and tumour necrosis factor-a (TNF-α) and serum fructosamine were determined, daily food and water intakes were measured and body weights were recorded every week. Also, we determined the total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), and the mRNAs expression of monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1 and CD36 mRNA in aorta. The results demonstrated that DSY had no direct effect in lowering serum fructosamine, but can produce a higher survival rate less body weight loss and more water intake in diabetic atherosclerosis in GK rats，which may be through anti-oxidation, anti-inflammation, regulating blood lipid, inhibiting expression of genes related to foam cell formation in rat aorta. Dan-Shen-Yin may be used as a promising agent for both prophylaxis and treatment of diabetic atherosclerosis.

Key words: Chinese medicine formula, Dan-Shen-Yin, diabetic atherosclerosis, oxidative stress, inflammation, blood lipid, genes related to foam cell formation, diabetic GK rats.

INTRODUCTION

Diabetic atherosclerosis is a major complication of diabetes, representing the predominant cause of morbidity and mortality in diabetic patients (Juutilainen et al., 2008; Zhang et al., 2006a, 2006b; Beverly et al., 2003). It is well known that oxidative stress (Nickenig et al., 2002; Witztum et al., 1991), inflammation (Kaneto et al., 2010; Koïtka et al., 2010), hyperlipidemia (Kearney et al., 2010; Murakami et al., 2010), hyperglycaemia (DCCTRG, 1993; Stratton et al., 2000), particularly the foam cell formation (Qiu et al., 2010; Yuri, 2006), participate in the pathophysiology of atherosclerosis. As a result, pharmacological inhibition of oxidative stress, inflammation, hyperlipidemia, hyperglycaemia and foam cell formation has been proposed as a strategy for reducing atherosclerosis. Pharmacological agents including statin (Jukema et al., 1995; Jenkins et al., 2005), ACE inhibitors (ACEI) (Ambrosioni et al., 1992; Cunha et al., 2005), aspirin (Guo et al., 2006; Paul et al., 2000), clopidogrel (Li et al., 2007; Husted et al., 2007) which can anti-atherosclerosis through inhibiting the 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA)

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(Jukema et al., 1995; Jenkins et al., 2005), blocking the conversion of the prohormone angiotensin I to the active hormone AII (Ambrosioni et al., 1992; Cunha et al., 2005), anti-platelet (Guo et al., 2006; Paul et al., 2000; Li et al., 2007; Husted et al., 2007) respectively, but only through the combination of them, can they have an better anti-atherosclerosis effect.

Recently, it has been proposed that a better way of treating complex diseases such as atherosclerosis disease maybe to aim at several targets (Zhang et al., 2010). Polypharmacology may provide a solution in this field (Frantz, 2005). Traditional Chinese medicines (TCMs) consist of several types of medicinal herb or mineral, and multiple components can hit multiple targets and exert synergistic therapeutic effects (Wang et al., 2010). Dan-Shen-Yin (DSY) is a famous traditional Chinese formula comprising Salvia Miltiorrhiza, Sandalwood and Fructus Amomi widely used in diabetic (Huang, 2007; Hu, 2009) and coronary heart disease (CHD) (Xie, 2008; Liu, 2010) clinic practice in Traditional Chinese Medicine (TCM) as a basic recipe and has produced a favorable effect (Huang, 2007; Hu, 2009; Xie, 2008; Liu, 2010). Recent pharmacological studies indicated that DSY had a role of anti-atherosclerosis in atherosclerosis rabbit model (Huang et al., 2002). Thus, we speculate that DSY may prevent atherogenesis in the process of diabetes. However, no research has been conducted on this. Therefore, our study was designed to focus on the effects of DSY on oxidative stress, inflammation, blood lipid and expression of genes related to foam cell formation in the early stage of atherosclerosis induced by L-NAME plus high-fat diet in diabetic GK rats (Zhang et al., 2006a, b).

MATERIALS AND METHODS

Preparation of DSY decoction

All the crude drugs of DSY including 120 g of Salvia Miltiorrhiza., 18 g of Sandalwood, 18 g of Fructus Amomi. were purchased from LBX pharmacy (Zhengzhou, China). To keep the consistency of the herbal chemical ingredients, all of the herbal components were originally obtained from the standard native sources as stated in the foregoing with GAP grade and the drugs were extracted with standard methods according to Chinese Pharmacopeoia (China Pharmacopoeia and Committee, 2000). Voucher specimens (No. 100322) had been kept. The mixture of DXY was made through boiling with distilled water at 100ºC for 30 min twice and the drug solution was vacuum cool-dried and made into drug powder and dissolved with distill water with the final concentration of 2.5 g/ml (equivalent to dry weight of raw materials).

Experimental design

As previously described (Zhang et al., 2006a; Zhang et al., 2006b), thirty GK rats (random blood glucose>11.1mmol/l) were randomly assigned to three groups (n = 10 in each group): early phase of diabetic atherosclerosis (DA)group, low dosage of DSY treated group (DA+DSYL, 5 g/kg/day intragastric administration of DSY), that is, the rat is 6.7 times the doses of human. It is consistent with literature (Pinkel, 1958). And high dosage of DSY treated group (DA+DSYH, 10 g/kg/day intragastric administration of DSY). The three groups of rats were all induced early phase of atherosclerosis by L-NAME in drinking water (1 mg/ml) plus high-fat diet (Usui et al., 2000). Ten Wistar rats used as non-diabetic control (Wistar group) were given sterile water and standard rat chow. The administration of DSY lasted for 4 weeks. In the end of the experiments, the serum level of malondialdehyde (MDA), total antioxidant capacity(T-AOC), superoxide dismutase (SOD) and GSH-peroxidase (GPX) activities and the serum level of glutathione(GSH) were measured (Qin et al., 2009). The concentrations of circulating inflammatory markers C-reactive protein (CRP), interleukin-6 (IL-6) and tumour necrosis factor-a (TNF-a) and serum fructosamine were determined, daily food and water intakes were measured and body weights were recorded every week (Kaneto et al., 2010; Koïtka et al., 2010). Also we determined the total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), and the mRNAs expression of monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1 and CD36 mRNA in aorta using real-time RT-PCR methods (Zhang et al., 2006a, b). The relative gene expression levels were obtained using the $2^{-\Delta\Delta Ct}$ method and normalized to the β-actin values.

Statistical analysis

Data are expressed as mean±S.D. Statistical analysis was performed with one-way ANOVA followed by t Tukey's post hoc test (Sonne et al., 2008) for multiple comparisons by using the SPSS 17.0 (SPSS Inc. Chicago, USA) for windows statistical package. A probability of less than 0.05 was considered to be statistically significant.

RESULTS

At the end of the experiment, 3 out of 10 rats in DA group died (n = 7), while the rats in other groups all survived (n = 10).

Effect of DSY on body weights, daily food and water intakes

During the experiment, the body weight of Wistar group rats significantly increased, however, all diabetic atherosclerosis rats displayed little or even no gain in their body weights. Interestingly, the body weight was significantly higher in the DA+DSYH group than that in the DA group after 2 weeks of treatment of DSY ($P<0.05$ or 0.01) (Figure 1A). The mean daily food intakes in each week by DA, DA+DSYL, and DA+DSYH rats all decreased significantly beyond our expectation compared to that by Wistar rats over the 4-week treatment period ($P<0.01$) as shown in Figure 1B. At 0 week, the water intakes (DA = 55.3±1.49 g/d, DA+DSYL = 60.2±1.93 g/d, DA+DSYH = 53.5±1.58 g/d) by GK rats were all significantly higher than that by Wistar rats (28.3±1.16 g/d). At the first week, the water intakes by all diabetic atherosclerosis rats (DA = 34±1.41 g/d, DA+DSYL= 35.1±2.02 g/d, DA+DSYH = 33±1.83 g/d) decreased nearly to the level of the Wistar rats (29.3±1.25 g/d),

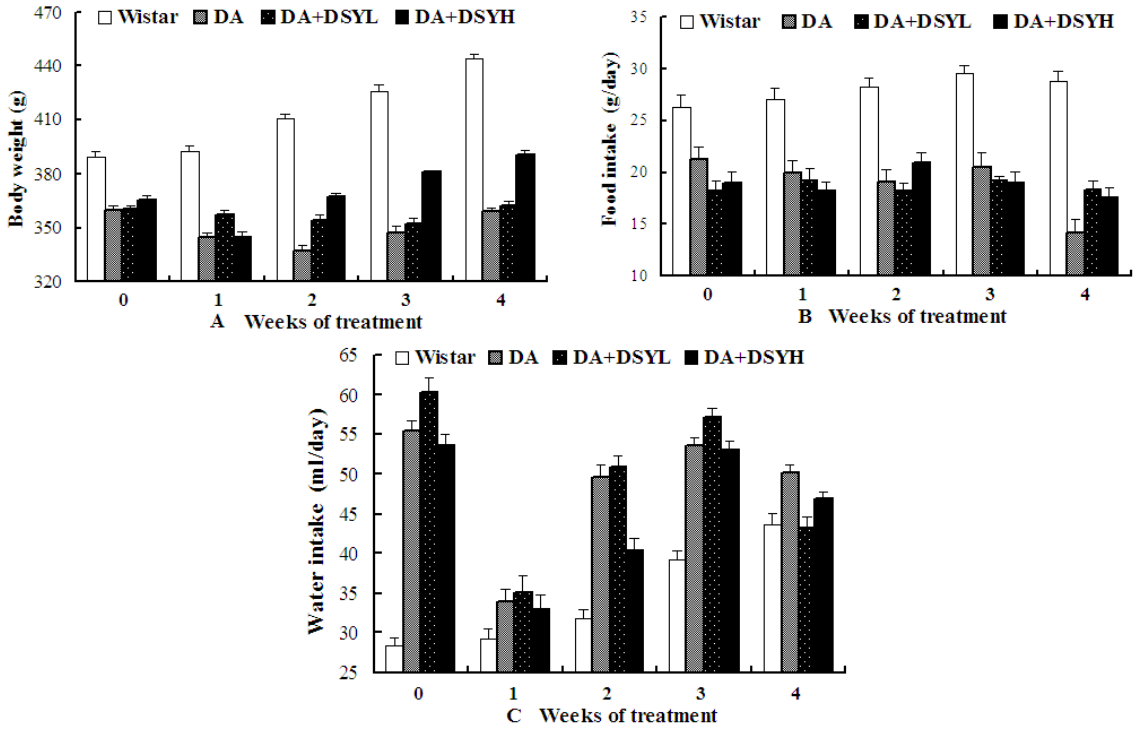

Figure 1. Body weight (A), food intake (B), and water intake (C) in the Wistar group (n = 10), DA group (n = 10 in the first week, n= 9 during the 2–3 weeks, n = 7 in the fourth week), DA + DSYL group (treated with DSY at a dose of 5 g/kg/day, n = 10), and DA + DSYH group (treated with DSY at a dose of 10 g/kg/day, n = 10).

Table 1. Effects of DSY on blood total cholesterol (TC), triglycerol (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) in the early stage of diabetic atherosclerosis rats**.** $^{*}P$<0.01 vs. Wistar group; $^{\#}P$<0.05 and $^{\#\#}P$<0.01 vs. DA group.

Group	n	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Wistar	10	0.462±0.023	1.711±0.024	1.174±0.026	0.459±0.037
DA	7	0.65±0.028*	2.308±0.027*	0.68±0.018*	0.505±0.025
DA+DSYL	10	0.625±0.016$^{*,\#}$	2.262±0.062*	1.095±0.026$^{*,\#\#}$	0.605±0.02$^{*,\#}$
DA+DSYH	10	0.614±0.021$^{*,\#\#}$	2.19±0.054$^{*,\#\#}$	1.115±0.035$^{*,\#\#}$	0.647±0.019$^{*,\#}$

went up again higher than that by Wistar rats (P<0.05 or 0.01) during the second and third weeks, decreased and shown no significant difference compared to that by Wistar rats during the last week (Figure 1C).

Effect of DSY on blood lipid

As shown in Table 1, TG (DA = 0.65±0.028 mmol/L, DA+DSYL = 0.625±0.016 mmol/L, DA+DSYH = 0.614±0.021 mmol/L), TC (DA =2.308±0.027 mmol/L, DA+DSYL = 2.262±0.062 mmol/L, DA+DSYH = 2.19±0.054 mmol/L), LDL-C (DA = 0.50±0.025 mmol/L, DA+DSYL = 0.605±0.02 mmol/L, DA+DSYH = 0.647±0.019 mmol/L) level in the early phase of diabetic atherosclerosis rats were higher than that in Wistar group (TG = 0.462±0.023 mmol/L,TC = 1.711±0.024 mmol/L,LDL-C = 0.459±0.037 mmol/L) (P<0.01). HDL-C level. (DA = 1.174±0.026 mmol/L, DA+DSYL = 1.095±0.026 mmol/L, DA+DSYH = 1.115±0.035 mmol/L) in DA, DA+ DSYL and DA+DSYH groups were lower than that in Wistar group (HDL-C = 1.174±0.026 mmol/L) (P<0.01).

There were no differences in TG, TC, and LDL-C, HDL-C level between DA + DSYL and DA + DSYH groups. However, compare to DA group, the TG, TC, LDL-C concentrations in DSY treated rats (DA+DSYL and DA+DSYH) were higher than that in DA rats (P<0.05 or 0.01), the HDL-C concentration in DSY treated rats (DA+DSYL = 1.095±0.026mmol/L,DA+DSYH = 1.115±0.035 mmol/L) were higher than that in DA rats (0.68±0.018 mmol/L) (P < 0.01).

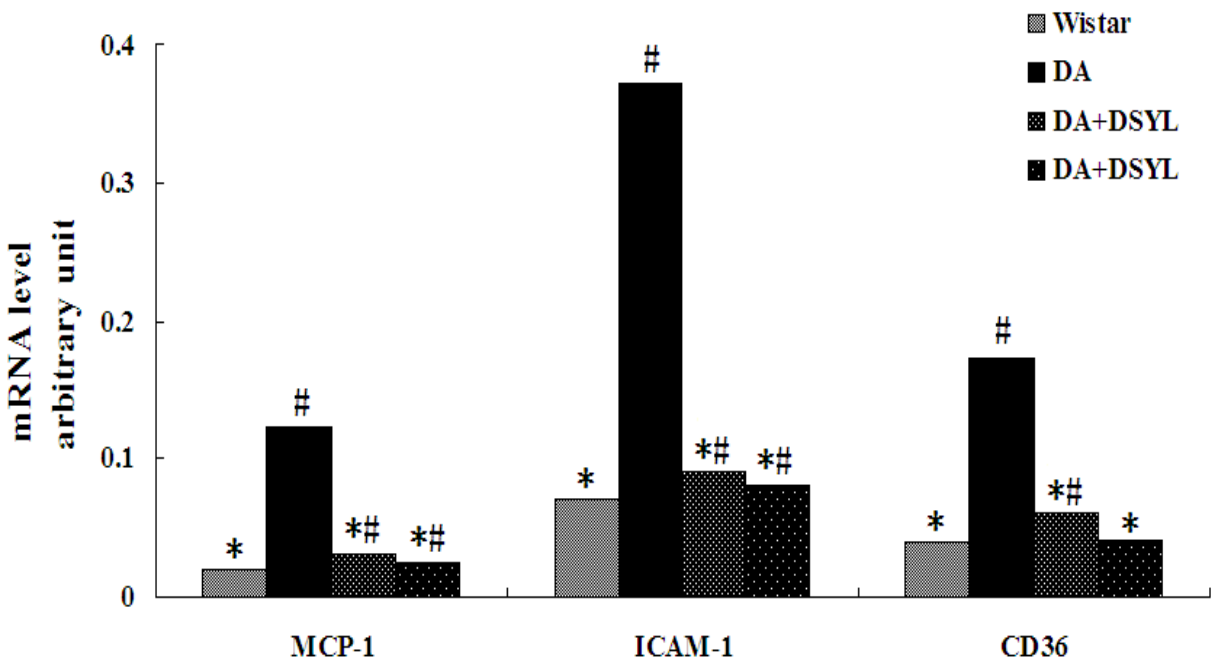

Figure 2. The Effects of DSY on MCP-1, ICAM-1 and CD36 mRNA level in aorta in the Wistar (n = 10), DA (n = 7), DA+DSYL (n = 10) and DA + DSYH (n = 10) group. *P<0.01 vs. DA group,# P<0.01 vs. Wistar group.

Effect of DSY on expression of genes related to foam cell formation

The MCP-1, ICAM-1 and CD36 mRNA expressions in aorta in the early phase of diabetic atherosclerosis rats increased steadily and significantly compared with these in Wistar rats (P<0.01). Treatment with DSY in early diabetic atherosclerosis rats resulted in significant inhibition of MCP-1, ICAM-1 and CD36 mRNA expressions (P<0.01) (Figure 2). Importantly, there were no differences in CD36 mRNA expressions between Wistar and DA+DSYH groups.

Effect of DSY on antioxidant assay

The serum levels of T-AOC, SOD, GPX and GSH were significantly increased (16.23±2.38, 186.93±19.85, 418.72±29.96 U/ml and 212.25±31.58 mg/l, respectively), while the serum MDA level was significantly decreased (6.45±2.56 U/ml), compared with the normal controls and sham-operated rats (Figure 3). DSY at doses of 5 and 10 g/kg significantly reduced the serum levels of T-AOC (to 25.2±1.48 and 27.1±1.67 U/ml, respectively), SOD (to 210.4±3.2 and 222.7±10 U/ml, respectively) and GSH (to 271.3±6.63 and 277.8±5.41 mg/l, respectively) (all P<0.01), (Figure 3A, C and E). In addition, DSY at a dose of 10g/kg increased the serum GPX level (to 461.3±2.71 U/ml, P<0.01) (Figure 3D), but at a dose of 5g/kg decreased the serum MDA level (to 4.03±0.18 U/ml, P<0.01) (Figure 3B).

Effect of DSY on fructosamine and circulating inflammatory markers

At the end of experiment, the serum levels of fructosamine (mmol/L) in DA (4.05±0.17), DA+DSYL (3.96±0.2), and DA+DSYH (3.87±0.13) rats were significantly higher than that in Wistar rats (3.02±0.17, P<0.01). DSY treatment did not decrease the serum concentrations of fructosamine compared with that in DA rats (P>0.05). The serum levels of CRP (mg/L) in DA(722.4±39.98), DA+DSYL (643.2±45.22), and DA+DSYH (628.4±36.52) groups were higher than that in Wistar group (360.6±38.03,P<0.01).The CRP concentrations in DSY treated groups were decreased compared with that in DA group (P<0.01). TNF-α levels (pg/ml) in DA rats (2.314±0.16) were higher compared to that in Wistar rats (1.622±0.23, P<0.01). However, treatment with DSY (2.13±0.23 in DA+DSYL group and 2.046±0.18 in DA+DSYH group) significantly decrease the serum TNF-α concentrations (P<0.05 or 0.01 versus DA group) to a level that was not different from that seen in Wistar group. While IL-6 levels (pg/ml) in DA rats (208.5±9.14) were higher compared to that in Wistar rats (93.1±4.61, P<0.01). However, treatment with DSY(140.6±8.21 in DA+DSYL group and 122±4.55 in DA+DSYH group) significantly decrease the serum IL-6 concentrations (P< 0.01 versus DA group).

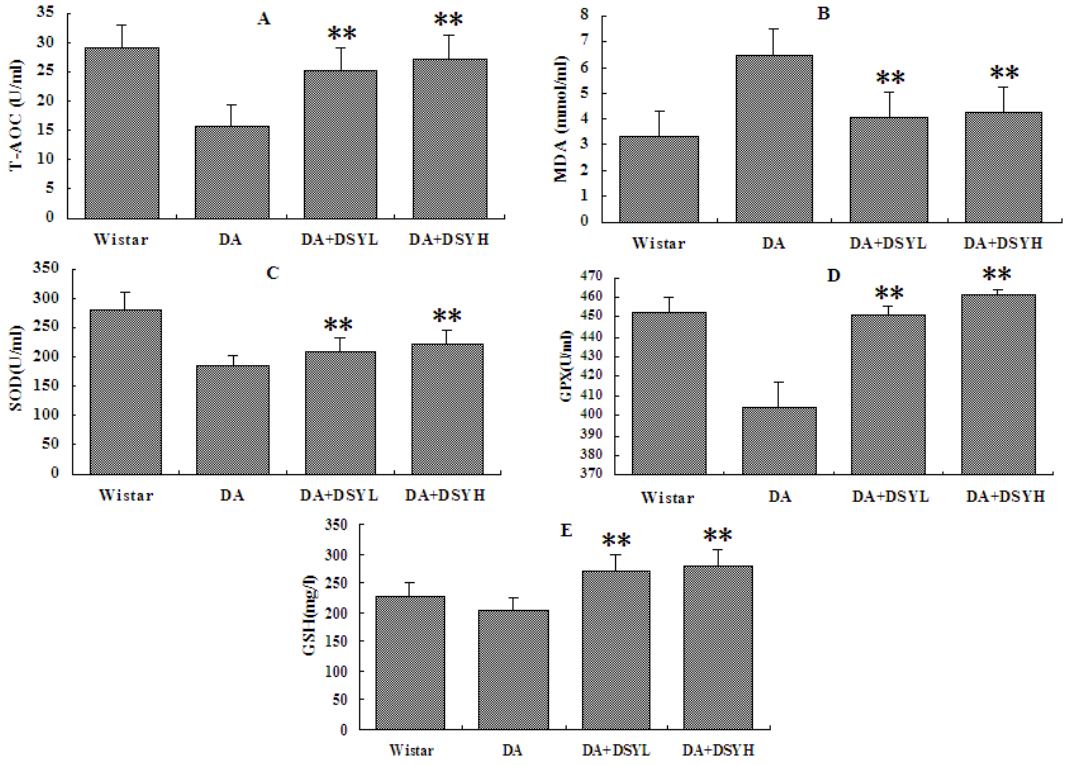

Figure 3. Effect of DSY on antioxidant assay: (A) total antioxidant capacity (T-AOC); (B) malondialdehyde (MDA); (C) superoxide dismutase (SOD); (D) glutathione-peroxidase (GPX) and (E) glutathione (GSH). Wistar group (n=10),DA group (orally given vehicle, n=7), DA+DSYL (orally given DSY extract 5 g/kg, n=10) and DA+DSYH (orally given DSY extract 10g/kg, n=10). **p<0.01 vs. DA group.

DISCUSSION

At the end of experiment, 3 out of 10 rats in DA group died, while that in other groups all survived. This is the most direct evidence that diabetic atherosclerosis has a high mortality risk and DSY has a protective role. Further studies found that DSY produced less body weight loss, decreased water intake, lowered TG, TC and LDL-C, increased HDL-C, inhibit the genes expression of MCP-1, ICAM-1 and CD36, decreased the concentrations of CRP, TNF-a and IL-6, reduced the amounts of MDA, but enhanced T-AOC, SOD, GPX activities and GSH content in diabetic atherosclerosis rats, but had no direct effect in lowering serum fructosamine. It is well established that type 2 diabetes frequently has an abnormal blood lipid profile consisting of normal or slightly elevated low-density lipoprotein cholesterol, moderately decreased high-density lipoprotein cholesterol, and high triglycerides (Zhang et al., 2006a; Zhang et al., 2006b). Interestingly, the present study demonstrated that serum TG, TC and LDL-C were significantly lower whereas the HDL-C was higher in the DSY-treated group than that in DA group. Significant lowering of TG, TC, LDL-C and rise in HDL-C is a very desirable biochemical state for prevention of atherosclerosis (Wilson et al., 2010). Therefore, our results showed that DSY had some protective effects against diabetic lipid metabolism abnormality. In another hand, DSY had no effect on blood glucose (data not shown).

However, it showed a regulation role on blood lipid. So DSY had the direct effect on blood lipid, but not the result of decreasing hyperglycemia. In addition, DSY-treated rats showed higher food intakes and less body weight loss. As well known that foam cell is a crucial landmark for the pathogenesis of atherosclerosis, and macrophage-derived foam cell formation is an early event in the onset of atherosclerosis (Baker et al., 2011; Steinberg et al., 2010; Angheloiu et al., 2011; Bai et al., 2011). The population of macrophages in the arterial wall highly depends on monocyte infiltration from the blood circulation (Woollard et al., 2010; Tian et al., 2010; Keul et al., 2011). MCP-1 and ICAM-1 play key roles in the process of monocyte transmigration to the subendothelial space (Huo et al., 2001; Arakawa et al., 2010; Rautou et al., 2011; Missiou et al., 2010). After monocyte transmigration and differentiation to macrophages in the subendothelial space, macrophages expressing scavenger receptor-A and CD36 uptake modified

LDL particles and gradually become foam cells (lipid-laden macrophages) (Curat et al., 2004; Randolph et al., 1998; Ley et al., 2011). Data from murine models of atherosclerosis have demonstrated that oxidized low-density lipoprotein (ox-LDL) uptake was primarily mediated by CD36 (Park et al., 2009; Silverstein., 2009; Mäkinen et al., 2010; Kunjathoor et al., 2002; Nicholson., 2004). Thus, regulating the expression of these genes, including MCP-1, ICAM-1 and CD36, is crucially important for the initiation of a lesion in the arterial wall. The inhibition of foam cell formation will be important in the prevention of atherogenesis in the process of type 2 diabetes. Our results showed that MCP-1, ICAM-1 and CD36 expression in aorta in the early phase diabetic atherosclerosis rats were significantly higher than that in Wistar control rats. Administration of DSY could downregulate the expression of MCP-1, ICAM-1 and CD36, the key genes related to foam cells formation. Therefore, we speculated that DSY had the effect of Anti-atherosclerosis in diabetes. Previous literature have found that CRP (Hu et al., 2009; Lee et al., 2009), TNF-α (Schram et al., 2003; Zhang et al., 2009) and IL-6 (Saremi et al., 2009; Andriankaja et al., 2009) are strong predictors of increased risk for T2DM and atherosclerosis which can reflect low-grade inflammation and vascular injury. Our results clearly showted that DSY could decrease the circulating levels of CRP, TNF-α and IL-6 in diabetic atherosclerosis rats. Fructosamine could reflect blood glucose changes for 2-3 weeks and not be affected by diet (Innes et al., 2011). The concentrations of fructosa-mine in DSY-treated group were not lower than that of diabetic atherosclerosis group. This showes that DSY had no direct effect on blood glucose, but could decrease the level of inflammatory markers. From this perspective, the decreased level of circulating inflammatory marker by DSY was not the direct effects of hypoglycemia.

Evidences gathered from systematic reviews demonstrate that Oxidative stress is suggested to be associated with macrovascular and microvascular diabetes complications and could thus be another possible pathogenetic link between atherosclerosis and diabetes (Helmersson et al., 2004; Keaney et al., 2003; Madamanchi et al., 2005). Literature data have indicated that glucose challenge (Mohanty et al., 2002) and atherosclerosis (Lee et al., 2010; Dandona et al., 2003) leads to increased generation of reactive oxygen species (ROS). However, ROS, which possess highly reactive and toxic properties, play an important role in the development of atherosclerosis and diabetes (Paravicini et al., 2008). In response to this, animals have developed a natural defensive system to cope with these unwanted and toxic species. Such defense mechanisms include superoxide dismutase (SOD), glutathione peroxidase (GPX) and others (Gilberto et al., 2008). The measure of total antioxidant capacity (T-AOC), which is considered the cumulative action of all the antioxidants present in serum, can provide an integrated parameter rather than the simple sum of measurable antioxidants (Ghiselli et al., 2000). The capacity of known and unknown antioxidants and their synergistic interaction is therefore assessed, thus giving an insight into the delicate balance in vivo between oxidants and antioxidants (Qin et al., 2009). SOD is a very important enzyme and its activity of SOD reflects the cellular capability of scavenging/quenching free radicals (Zheng et al., 2008). MalondialdehyDe (MDA), the degradation product of the oxygen-derived free radicals and lipid oxidation, can interfere with the metabolism of protein, glucose, and nucleic acid, which results in the decrease in activity of enzyme, template dysfunction of nucleic acid, and injury of tissues and cells (Gerster., 2008). Then the level of MDA is regarded as the degree of lipid peroxidation. In view of evaluating the basal metabolism of free radicals, the activity of SOD and the level of MDA are the principal pathophysiologic parameters. Glutathione being an important cellular reductant, involved in protection against free radicals, peroxides and toxic compounds (Gerster, 2008). Depletion of GSH is one of the primary factors that permit lipid peroxidation (Konukoglu et al., 1998). It is reformed from GSSG, and GPX is a key catalyzer (Lappalainen et al., 2009).

Our present study demonstrated that T-AOC of the DA group was a significant decrease compared to Wistar group, and showed that diabetic atherosclerosis increased oxidative stress. The results also demonstrated that DSY increases the serum level of T-AOC (Figure 3A). SOD and GPX are two critical enzymes of the known antioxidant, which may contribute to the increase of T-AOC. In the study, DSY also increased the serum levels of SOD when administered at doses of 5 and 10 g/kg but low MDA production (Figure 3B and C), which implies that the formula may affect the level of endogenous antioxidants or oxidative stress or both. One of the possible explanations is that elevated activities of SOD scavenged excessive ROS and attenuated the lipid peroxidation. However, no significant changes in GSH and GPX levels might signify that the level of oxidative stress was not severe enough for these compounds to be involved in the present study. However, the results showed that administration of DSY caused a significant increase in the GSH and GPX levels, which might be due to the treatment group containing compounds present in the DSY (Figure 3D and E).

So far, it is widely accepted that multiple constituents are responsible for the therapeutic effects of TCM, especially for treating complex illnesses such as diabetes and cardiovascular disease and water decoctions of DSY are commonly used in clinics in China. The study provides evidence for the first time to elucidate its integrated protective effects in diabetic atherosclerosis rat models for produce a higher survival rate, likely through anti-oxidation, anti-inflammation, regulating blood lipid, inhibiting expression of genes related to foam cell formation in rat aorta.

In conclusion, our results suggested that Dan-Shen-Yin had a beneficial effect on blood lipid, oxidative stress, inflammation, and inhibited the expression of those genes that are related to the formation of foam cell in early phase of diabetic atherosclerosis, and can produce a higher survival rate, less body weight loss, and decrease water intake in diabetic atherosclerosis in GK rats. Thus, it is valuable to develop this formula into a potential therapeutic reagent with modern pharmacological standard for diabetic atherosclerosis.

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