Anti-coagulatory and Anti-inflammatory Effects of Astaxanthin in Diabetic Rats

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Abstract

Astaxanthin at 0.01 or 0.05% of the diet was supplied to diabetic rats for 12 wks. Astaxanthin intake significantly increased its deposit in plasma, and retained glutathione content, reduced the production of reactive oxygen species, interleukin-6, tumor necrosis factor-α, and monocyte chemoattractant protein-1 in blood and kidney of diabetic rats (P<0.05). Astaxanthin treatments also significantly decreased plasma levels of C-reactive protein and von Willebrand factor in diabetic rats (P<0.05). Astaxanthin intake at 0.05% significantly diminished plasminogen activator inhibitor-1 and factor VII activities, enhanced antithrombin-III and protein C activities in circulation (P<0.05). These results support that astaxanthin could attenuate diabetes associated coagulatory, oxidative, and inflammatory stress.

Keywords: astaxanthin, diabetes, coagulation, reactive oxygen species

Introduction

The pathological characteristics of diabetes include hyperglycemia, cytokine imbalance, and coagulation predomination (Yamada and others 2000; Brownlee 2001). Coagulatory disorder often occurs in diabetic patients with poor glycemic control (Nwose and others 2009). Clinical studies revealed that the increase of coagulation factors such as factor VII (FVII), and decrease of anticoagulation factors such as antithrombin-III (AT-III) in circulation of diabetic patients led to hypercoagulability, which promoted the development of diabetic complications, especially vascular diseases (Myrup and others 1995; Asakawa and others 2000). Lemkes and others (2010) indicated that enhanced oxidative stress from hyperglycemia disturbed the balance between coagulation and anti-coagulation, which facilitated the occurrence of arterial and venous thrombotic events. It is reported that excessive production of reactive oxygen species (ROS), acting as signaling molecules in thrombogenic cycle, favors the progression of thrombosis (Herkert and others 2004). Thus, hemostatic imbalance warrants attention in order to avoid diabetic vascular diseases. In addition, the overproduced pro-inflammatory cytokines and chemokines such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1 augment systemic inflammatory stress, and exacerbate diabetic deterioration (Geerlings and others 2000; Drimal and others 2008). Therefore, any agent with anti-coagulatory and/or anti-inflammatory activities may potentially prevent or delay the development of diabetic hemostatic disorders.

Astaxanthin is a nonprovitamin A carotenoid naturally found in many marine foods such as algae, crustaceans, and fish. Naito and others (2004) reported that dietary intake of astaxanthin at 0.02% improved glycemic control in female db/db mice. Manabe and others (2008) indicated that this compound could scavenge ROS in high glucose treated mesangial

cells. Other studies also revealed that this compound could provide anti-oxidative protection for diabetic animals (Otton and others 2010; Marin and others 2011). In addition, our previous study found that astaxanthin exhibited neuroprotective effects against inflammatory damage in nerve growth factor differentiated PC12 cells (Chan and others 2009). Although these previous studies suggest that astaxanthin possesses anti-oxidative and anti-inflammatory activities, it remains unknown whether this compound is able to affect coagulation associated factors. It is hypothesized that astaxanthin could attenuate diabetes associated hemostatic disorder, at least via diminishing oxidative and/or inflammatory stress.

The major purpose of our present study was to examine the anti-coagulatory effects of astaxanthin at two doses in diabetic rats. The influence of this compound upon oxidative and inflammatory factors in diabetic rats was also evaluated. These results will enhance our understanding regarding the possible application of this compound against diabetic progression.

Materials and Methods

Chemicals

Astaxanthin (AX, 99.5%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used in these measurements were of the highest purity commercially available.

Animals and diets

Male Sprague-Dawley rats were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Rats were housed individually in stainless steel wire-bottom cages under controlled conditions of temperature (22 ± 2 °C), humidity

(50–60%) and light (lights on from 0700 to 1900). They were given a regular rat chow and water *ad libitum* for acclimatization. To induce diabetes, 8-wk old rats with body weight of 195-205 g were treated with a single i.v. dose (50 mg/kg BW) of streptozotocin dissolved in citrate buffer (pH 4.5) into the tail vein under a fasting state. Blood glucose level was monitored on day 10 from the tail vein using a one-touch blood glucose meter (Lifescan, Inc. Milpitas, CA, USA). Rats with fasting blood glucose levels ≥ 200 mg/dL were used for this study. All animals received humane care as outlined in the Chinese version of Guide for the Care and Use of Laboratory Animals, and approved by the Providence University Animal Care Committee.

Experimental design

After diabetes was induced, rats were divided into three groups (8 rats per group). AX of 0.01% was selected as an effective low dose according to our pretest and the study of Naito and others (2004), high dose was 5 times of the low dose. AX at 0.01 or 0.05 g was mixed with 99.99 or 99.95 g powder diet containing (g/100 g): 64 starch (as corn starch), 23 protein (as casein), 3.5 fat (as soybean oil), 5 fiber, 1 vitamin mixture, and 3 salt mixture (PMI Nutrition International LLC, Brentwood, MO, USA), and supplied to diabetic rats. Diet was freshly prepared everyday to prevent oxidation of AX. One group of rats without diabetes (normal) and with normal diet was used for comparison. All rats had free access to food and water at all times. Water intake, feed intake, and body weight were recorded. Plasma levels of glucose and insulin were measured at wks 1 and 12. After 12 wk supplementation, overnight-fasted rats were killed under anesthesia by intraperitoneal injection of sodium pentobarbital at 50 mg/kg BW. Blood and organs were collected and weighed. Plasma was separated from erythrocytes immediately. Kidney at 0.1 g was homogenized on ice in 2 mL

phosphate buffer saline (PBS, pH 7.2), and centrifuged at 10000 xg for 15 min at 4 °C. The resultant supernatant was used for measurements. The protein concentration of plasma or kidney homogenate was determined by the method of Lowry and others (1951) using bovine serum albumin as a standard. In all experiments, the sample was diluted to a final concentration of 1 g protein/L using PBS.

Blood analyses

Plasma glucose level (mg/dL) was measured by a glucose kit (Sigma Chemical Co., St. Louis, MO, USA). Plasma insulin level (μg/L) was determined by a rat insulin radioimmunoassay kit (Linco Research Inc., St. Charles, MO, USA). HOMA-IR was calculated according the equation: glucose (mM) × insulin (μIU/mL)/22.5. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured by commercial assay kits (Randox Laboratories Ltd., Crumlin, UK). Plasma blood urea nitrogen (BUN) and creatinine concentrations were detected by a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA, USA). Plasma AX level was analyzed by a HPLC method described in Park and others (2010), in which trans-β-apo-8'carotenal purchased from Sigma Chem. Co. (St. Louis, MO, USA) was used as the internal standard. Triglyceride (TG) and total cholesterol (TC) levels in plasma (mg/dL) were determined by commercial triglycerides and cholesterol kits (Boehringer Mannheim, Germany).

Determination of oxidative and anti-oxidative status

Malonyldialdehyde (MDA), an index of lipid peroxidation, was measured by a commercial assay kit (OxisResearch, Portland, OR, USA). Glutathione (GSH) concentration (nmol/mL or mg protein) was determined by a commercial colorimetric GSH assay kit (OxisResearch, Portland, OR, USA). The method described in Ali and others (1992) was

used to measure ROS level. Briefly, 10 mg sample was homogenized in 1 mL of ice cold 40 mM Tris–HCl buffer (pH 7.4), and further diluted to 0.25% with the same buffer. 2'7'-Dichlorohydrofluorescein diacetate (DCFH-DA) was deesterified in homogenate to dichlorohydrofluorescein, and then oxidized to fluorescent dichlorofluorescein by ROS. Homogenate at 100 μL was mixed with 100 μL DCFH-DA, and followed by incubating in a water bath for 30 min at 37 °C and centrifuging at 12,000 ×g for 8 min at 4 °C. Fluorescence was assayed by a fluorescence spectrometer with excitation at 488 nm and emission at 525 nm. ROS level was quantified from a standard curve of dichlorofluorescein.

Measurement of inflammatory and endothelial injury markers

The levels of IL-6, TNF-α, and MCP-1 were measured by cytoscreen immunoassay kits (BioSource International, Camarillo, CA, USA). Samples were assayed in duplicates according to manufacturer's instructions. Plasma levels of C-reactive protein (CRP) and von Willebrand factor (vWF) were also measured as inflammatory and endothelial injury markers. CRP (μg/mL) was determined with a commercial ELISA kit (Anogen, ON, Canada). vWF antigen level was measured by a rabbit anti-rat vWF polyclonal antibody (Dako, Glostrup, Denmark), and vWF level was expressed as relative percentage as compared to normal pooled plasma.

Measurement of coagulation and anticoagulation factors

Coagulation factor, FVII; anticoagulation factors, AT-III and protein C; fibrinolytic factor, plasminogen activator inhibitor-1 (PAI-1), were measured. Blood samples were anticoagulated using sodium citrate according to the protocols provided by the manufacturers. FVII activity was determined by a commercial kit (Coaset® FVII, Chromogenix, Lexington, MA, USA), which was positively correlated to the production of a yellow pigment

(p-nitroaniline), and the absorbance at 405 nm was assayed. The activity of AT-III and protein C in plasma was measured by commercial AT-III and protein C kits (Sigma Chemical Co., St. Louis, MO, USA), respectively. The standard curve for these two measurements was prepared by standard plasma. The activity of AT-III and protein C was expressed as a percentage related to the activity of standard plasma. PAI-1 activity (kU/L) was assayed by a commercial kit (Trinity Biotech plc., Bray, C. Wicklow, Ireland).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was done using one-way analysis of variance, and post-hoc comparisons were carried out using Dunnett's t-test. Statistical significance is defined as $P< 0.05$.

Results

Feed and water intake, body weight, and organ weight of normal and diabetic rats are presented in Table 1. Compared with diabetic control group, AX treatments did not affect water intake, feed intake, and body weight ($P>0.05$). However, AX intake significantly lowered liver and kidney weights in diabetic rats ($P<0.05$). Plasma levels of AX, glucose, insulin, and HOMA-IR are shown in Table 2. Compared with diabetic control group, AX treatments significantly increased plasma AX level ($P<0.05$); but failed to affect glucose, insulin, and HOMA-IR levels ($P>0.05$).

Activity of ALT and AST, and level of TG, TC, BUN, and creatinine are presented in Table 3. Diabetic rats had significantly higher ALT and AST activities, and TG, TC, BUN, and creatinine levels than normal rats ($P<0.05$). However, AX treatments significantly decreased these parameters when compared with diabetic control group ($P<0.05$). Effects of

AX on MDA, ROS and GSH levels in plasma and kidney are shown in Table 4. AX intake significantly lowered diabetes induced increase in MDA and ROS levels, as well as retained GSH content ($P<0.05$).

Effects of AX on plasma coagulation and anticoagulation factors are shown in Figures 1 and 2. AX treatments at 0.01 and 0.05% significantly declined vWF level, and enhanced protein C activity ($P<0.05$). But this agent only at 0.05% reduced FVII and PAI-1 activities, and elevated AT-III activity ($P<0.05$). As shown in Table 5, diabetes significantly increased the levels of CRP, IL-6, TNF-α, and MCP-1 in plasma and kidney ($P<0.05$); and AX treatments significantly decreased the release of these inflammatory factors in plasma and kidney ($P<0.05$).

Discussion

Based on our data of glucose, insulin, and HOMA-IR, astaxanthin at 0.01 or 0.05% was not an effective agent for glycemic control in male Sprague-Dawley rats. However, Naito and others (2004) reported that astaxanthin at 0.02% improved hyperglycemia in female db/db mice. The different response to this compound from that previous and our present studies might be due to the etiology and sex between these two diabetic models. Even so, we found astaxanthin supplement increased its deposit in plasma, lowered lipid accumulation in circulation, improved hepatic and renal functions, attenuated renal oxidative and inflammatory stress, as well as exhibited substantially anti-coagulatory effects in diabetic rats. These findings still suggest that this compound could provide multiple protective actions against diabetic deterioration.

PAI-1 is the primary physiologic inhibitor of fibrinolysis (Urano and others 2000).

vWF is involved in platelet adhesion and aggregation (Kessler and others 1998). FVII is the first enzyme in blood coagulation system for triggering the clotting cascade (Eigenbrot and Kirchhofer 2002). Thus, the elevated level or activity of FVII, vWF, and PAI-1 as we observed in those diabetic rats reflected a disruption in the balance of factors regulating coagulation and fibrinolysis, and obviously promoted the progression of thrombosis. However, we found that astaxanthin supplement at 0.05% lowered vWF level, and declined PAI-1 and FVII activities, which contributed to attenuate coagulatory disorder. It is known that hyperglycemia induced oxidative stress causes endothelial dysfunction, and enhances the release of PAI-1 and vWF (Lee et al., 2005). Furthermore, ROS could stimulate platelet hyperactivity, and facilitate coagulation (Ceriello and others 1995; Meerarani et al., 2006). Thus, lowering ROS production could benefit hemostatic balance. The anti-oxidative property of astaxanthin under diabetic condition has been observed (Manabe and others, 2008; Marin and others, 2011), and those authors reported that this compound could decrease ROS generation, and improve redox imbalance. The results of our present study revealed that the intake of this compound at test doses effectively reduced plasma and renal production of ROS and MDA, as well as retained GSH content in circulation and kidney. These findings agreed that astaxanthin via its anti-oxidative activity alleviated redox disorder in circulation and kidney of diabetic rats. Thus, it is highly possible that astaxanthin diminished oxidative stress, which consequently reversed the imbalance of hemostatic factors, and abated hyper-coagulatory risk. In addition, AT-III and protein C are anticoagulation factors because AT-III inhibits the activity of several proteases in the coagulation cascade, and protein C inactivates coagulation factors such as FVII (Asakawa and others 2000). Our present study found that astaxanthin treatments elevated AT-III and protein C activities. Apaprently, the

anti-coagulatory effects of this agent could be partially ascribed to it enhance thrombolysis. These findings suggested that astaxanthin was able to reduce the risk of diabetes associated atherogenesis and thrombosis.

Inflammatory injury is another contributor for diabetic deterioration (Chao and others 2010). The overproduction of IL-6 and TNF-α exacerbates the severity of diabetes because these cytokines are central mediators for the progression of inflammation, endothelial dysfunction, and hypercoagulation (Navarro-González and Mora-Fernández 2008; Lin and others 2009). MCP-1 is a chemotactic factor for activating monocytes and macrophages, and could recruit monocytes to the sites of injury (Hatanaka and others 2006). Thus, lowering these cytokines and chemokines could ameliorate inflammation, and improve endothelial functions. Our present study found that astaxanthin supplement substantially decreased IL-6, TNF-α, and MCP-1 levels in circulation and kidney. These results supported that this compound was a potent agent against diabetes associated systemic and renal inflammatory injury. It is reported that ROS could stimulate the expression of inflammatory cytokines, and promote diabetes related inflammatory disorders (Takaishi and others 2003; Delmastro and Piganelli, 2011). Thus, diabetic inflammatory stress could be declined through suppression of ROS. Our data revealed astaxanthin intake effectively decreased ROS production in kidney and circulation, which consequently reduced the release of inflammatory cytokines and MCP-1. vWF is an endothelial injury biomarker, and increased vWF in diabetic individuals reflects the deterioration of endothelial dysfunction and inflammation (Vischer 2006). We notified astaxanthin intake lowered plasma vWF level in diabetic rats. It seems this compound also benefited endothelial functions. In addition, TNF-α and IL-6 could enhance the formation of PAI-1, and facilitated thrombosis (Aso and other 2003; Palomo and others

2006). Thus, the anti-coagulatory effect from astaxanthin might be partially ascribed to it diminish the generation of these cytokines.

In summary, astaxanthin at 0.05% markedly retained GSH, and reduced the formation of inflammatory cytokines, MDA, and ROS in circulation and kidney. Furthermore, this compound mitigated hemostatic disorder via decreasing FVII and PAI-1 activities, as well as increasing protein C and AT-III activities in circulation. These findings support that the supplement of this agent or foods rich in this compound might be helpful for the prevention or alleviation of diabetic complications.

Acknowledgement

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Table 1-Water intake (WI, mL/rat/d), feed intake (FI, g/rat/d), body weight (BW, g/rat) and organ weight (g/100 g BW) of normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 1 and 12. Data are mean ± SD, n=8.

	normal	DM	DM+AX, 0.01%	DM+AX, 0.05%
WI				
1	32.0±4.8	36.5±7.1	33.8±5.2	30.4±2.9
12	35.5±5.9	231.8±19.3[a]	238.2±30.3[a]	236.1±26.9[a]
FI				
1	22.4±2.1	24.8±1.8	20.3±2.5	21.7±2.6
12	27.2±1.6	60.4±6.2[a]	57.6±7.0[a]	51.3±6.3[a]
BW				
1	203.1±6.6	197.0±9.8	190.5±7.2	192.5±12.3
12	478.3±28.7	346.3±37.4[a]	355.7±40.6[a]	370.8±32.9[a]
Organ weight				
Liver	3.05±0.19	4.68±0.31[a]	4.11±0.23[a,b]	4.25±0.39[a,b]
Spleen	0.14±0.02	0.18±0.03	0.17±0.03	0.17±0.02
Kidney	0.52±0.03	1.19±0.11[a]	0.88±0.07[a,b]	0.86±0.10[a,b]

[a] $P < 0.05$ versus normal group.

[b] $P < 0.05$ versus DM group.

Table 2-Plasma AX (μg/L), glucose (mg/dL) and insulin (μg/L) levels and HOMA-IR in normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 1 and/or 12. Data are mean ± SD, n=8.

	normal	DM	DM+AX, 0.01%	DM+AX, 0.05%
Plasma AX				
wk 12	—*	—	3.2±0.4[a,b]	5.1±0.6[a,b]
Plasma glucose				
wk 1	101.4±6.1	241.8±9.5[a]	237.3±10.2[a]	244.7±13.0[a]
wk 12	96.8±8.2	360.4±21.2[a]	347.6±17.0[a]	335.3±25.3[a]
Plasma insulin				
wk 1	3.4±0.5	1.1±0.4[a]	1.0±0.3[a]	1.3±0.4[a]
wk 12	3.5±0.6	0.3±0.1[a]	0.3±0.2[a]	0.4±0.2[a]
HOMA-IR				
wk 12	1.0±0.3	6.4±1.1[a]	6.2±1.2[a]	5.7±0.9[a]

*Means too low to be detected.

[a]P <0.05 versus normal group.

[b]P <0.05 versus DM group.

Table 3-Activity of alanine aminotransferase (ALT, U/mL), aspartate aminotransferase (AST, IU/L), and levels of triglyceride (TG, mg/dL), total cholesterol (TC, mg/dL), , blood urea nitrogen (BUN, mg/dL) and creatinine (mg/dL) in plasma from normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 12. Data are mean ± SD, n=8.

	normal	DM	DM+AX, 0.01%	DM+AX, 0.05%
ALT	13.3±1.8	47.7±1.3[a]	27.6±2.7[a,b]	24.8±2.1[a,b]
AST	51.7±6.4	134.7±13.2[a]	84.6±8.2[a,b]	78.6±10.5[a,b]
TG	78.9±12.6	243.2±29.5[a]	83.7±9.8[b]	94.2±13.0[b]
TC	57.4±7.1	109.9±19.0[a]	72.1±10.2[b]	66.7±9.4[b]
BUN	13.2±1.2	26.9±2.6[a]	22.5±1.7[a,b]	20.7±0.9[a,b]
creatinine	3.5±0.9	13.8±2.5[a]	6.2±1.2[a,b]	6.3±1.4[a,b]

[a] $P < 0.05$ versus normal group.

[b] $P < 0.05$ versus DM group.

Table 4-Levels of MDA, ROS and GSH in plasma and kidney of normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 12. Data are mean ± SD, n=8.

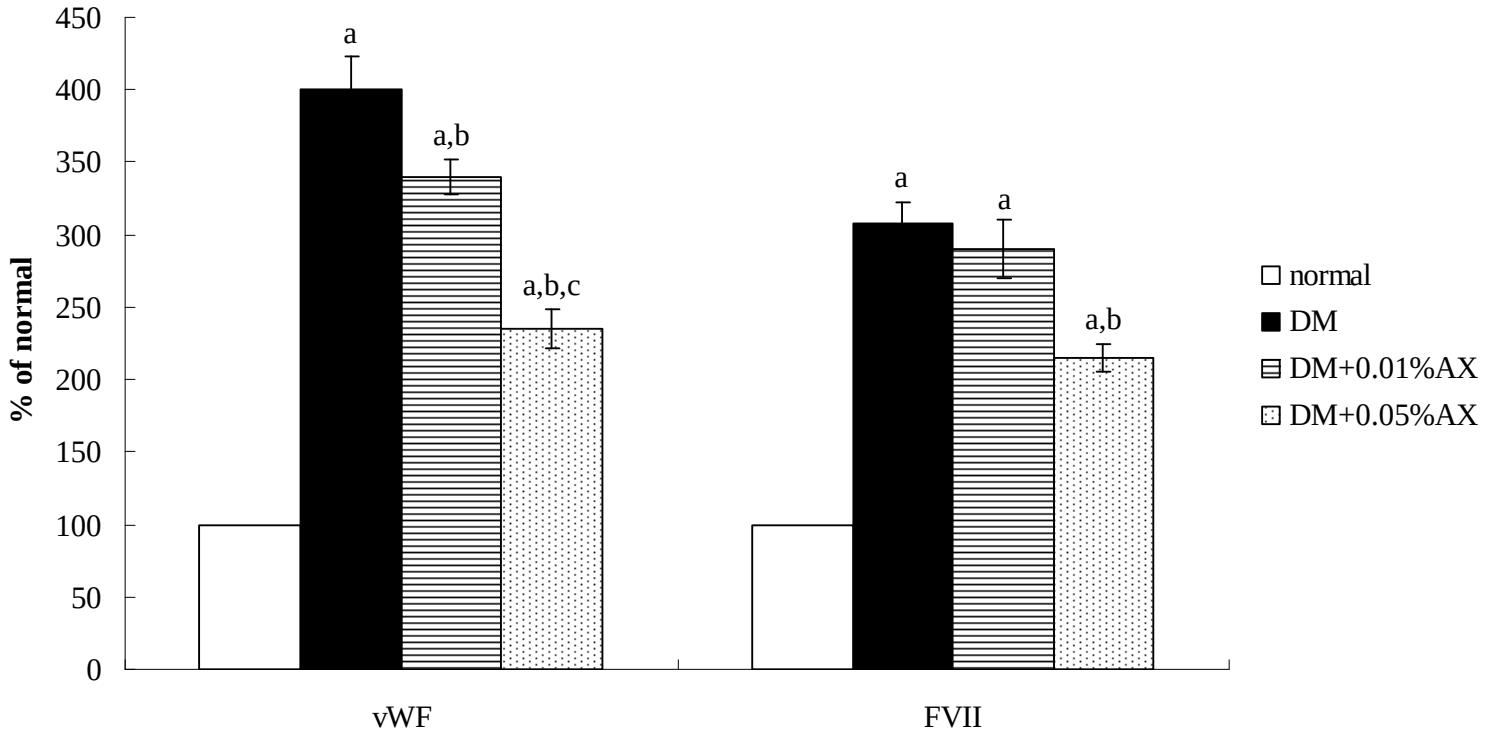
	normal	DM	DM+AX, 0.01%	DM+AX, 0.05%
Plasma				
MDA, μmol/L	2.6±0.1	5.3±0.2[a]	3.8±0.1[a,b]	3.4±0.2[a,b]
ROS, nmol/L	0.11±0.04	1.17±0.17[a]	0.81±0.10[a,b]	0.55±0.07[a,b,c]
GSH, μmol/L	91.5±4.6	30.3±8.2[a]	41.7±4.2[a,b]	55.6±5.8[a,b,c]
Kidney				
MDA, μmol/mg protein	3.2±0.4	6.4±0.9[a]	5.2±0.8[a,b]	5.0±0.5[a,b]
ROS, nmol/mg protein	0.19±0.06	1.46±0.21[a]	1.18±0.15[a,b]	0.78±0.10[a,b,c]
GSH, μmol/mg protein	0.75±0.14	0.21±0.06[a]	0.37±0.10[a,b]	0.51±0.08[a,b,c]

[a] $P < 0.05$ versus normal group.

[b] $P < 0.05$ versus DM group.

[c] $P < 0.05$ versus DM+AX, 0.01% group.

Figure 1-vWF level (%), FVII activity (%) and PAI-1 activity (kU/L) in plasma of normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 12. Data are mean ± SD, n=8. [a]*P* <0.05 versus normal group. [b]*P* <0.05 versus DM group. [c]*P* <0.05 versus DM+AX, 0.01% group.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DM+0.05%AX

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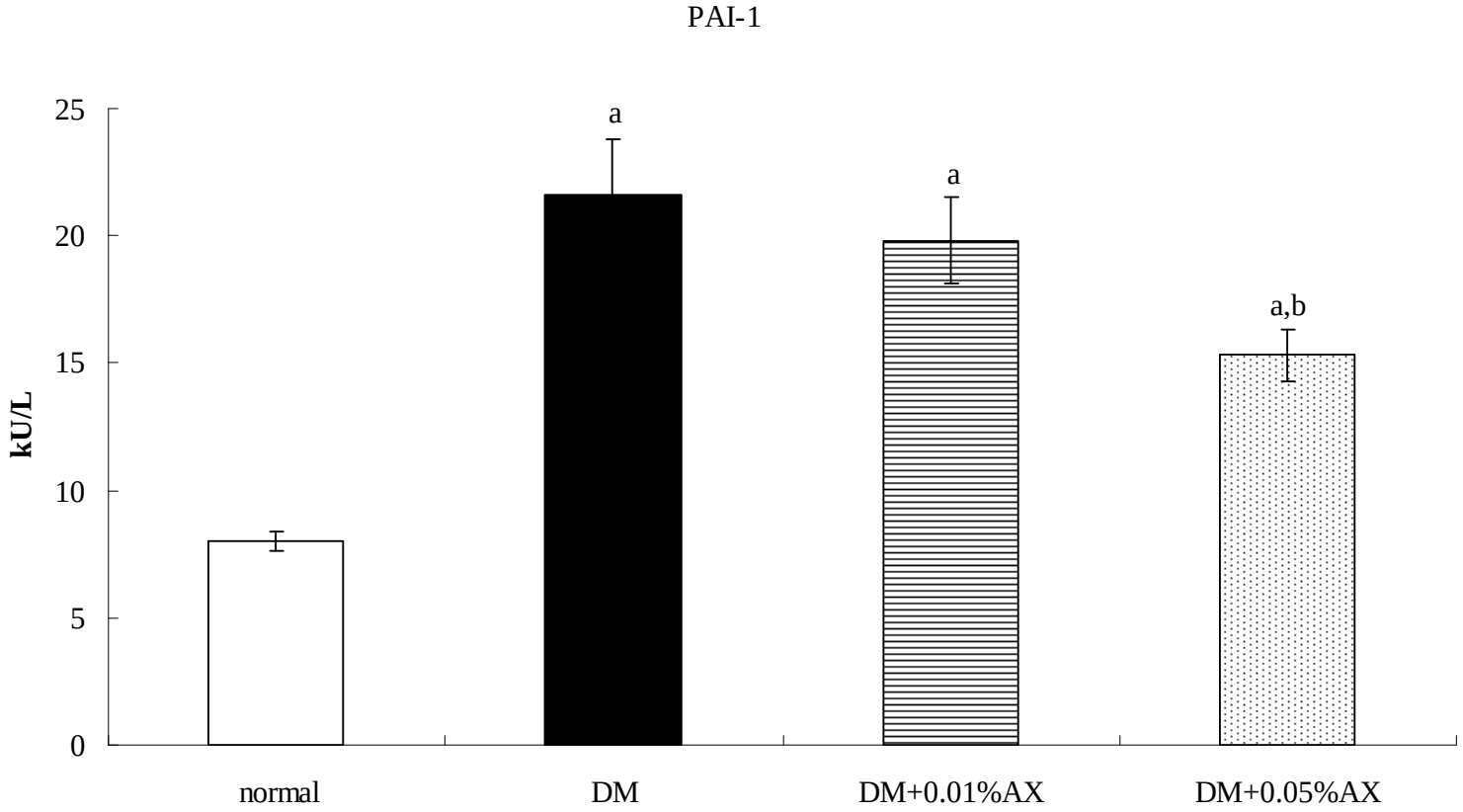

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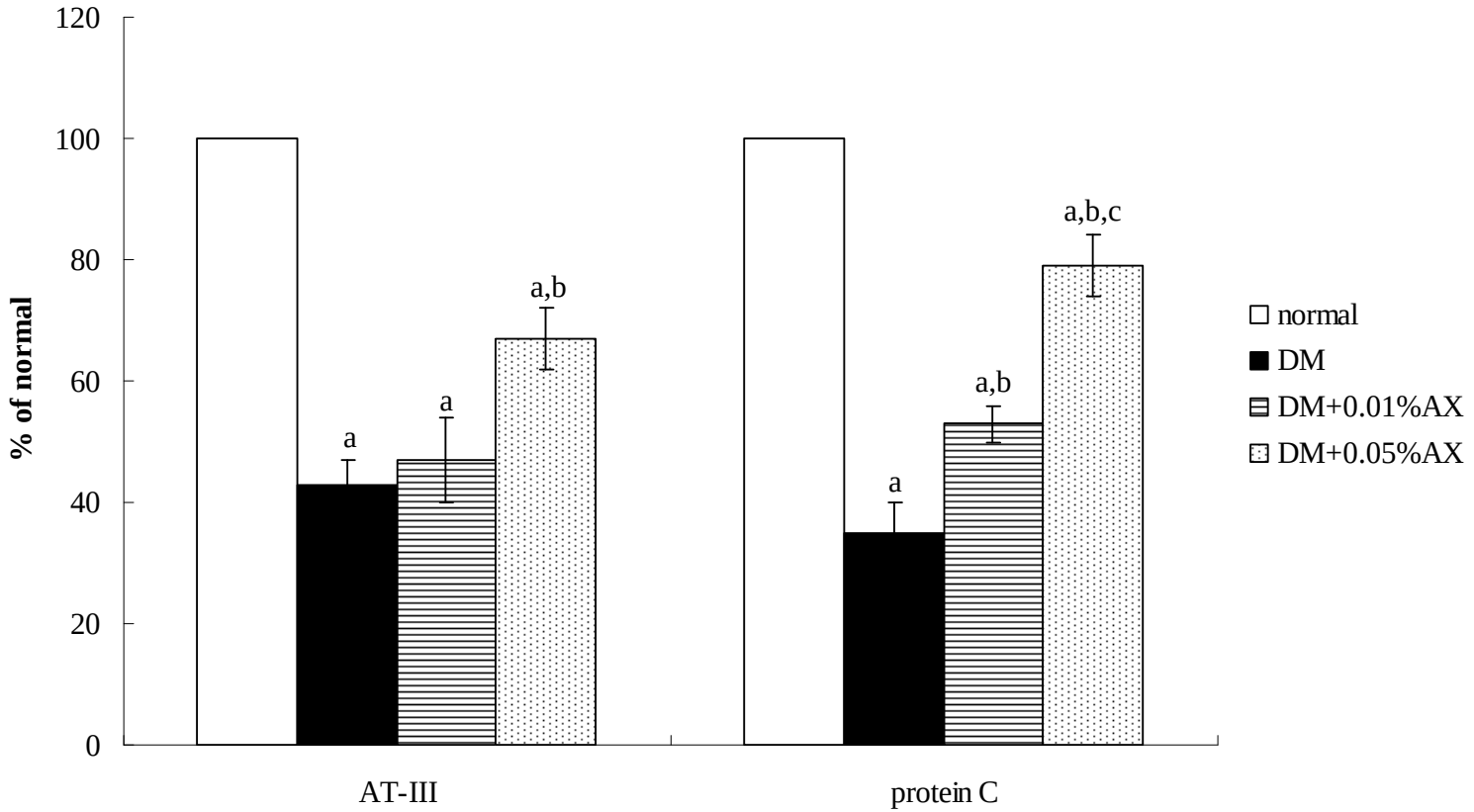
Figure 2-Activity of AT-III (%) and protein C (%) in plasma of normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 12. Data are mean ± SD, n=8. [a]*P* <0.05 versus normal group. [b]*P* <0.05 versus DM group. [c]*P* <0.05 versus DM+AX, 0.01% group.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DM+0.05%AX

1

Table 5-Levels of IL-6, TNF-α, MCP-1 and CRP in plasma and kidney of normal, diabetic rats consumed normal diet (DM), or 0.01, 0.05% AX at wks 12. Data are mean ± SD, n=8.

	normal	DM	DM+AX, 0.01%	DM+AX, 0.05%
Plasma				
IL-6, pg/mL	19±3	327±25[a]	252±19[a,b]	199±21[a,b,c]
TNF-α, pg/mL	46±9	438±31[a]	365±22[a,b]	267±18[a,b,c]
MCP-1, pg/mL	26±4	298±17[a]	223±20[a,b]	157±13[a,b,c]
CRP, μg/mL	0.62±0.11	1.70±0.14[a]	1.29±0.21[a,b]	0.98±0.13[a,b,c]
Kidney				
IL-6, pg/mg protein	13.6±1.2	108.4±9.5[a]	87.3±6.0[a,b]	78.4±7.2[a,b]
TNF-α, pg/mg protein	61±5	313±23[a]	187±17[a,b]	120±10[a,b,c]
MCP-1, μg/mg protein	21.0±1.6	58.4±3.3[a]	49.6±2.5[a,b]	44.5±2.8[a,b]

[a] P <0.05 versus normal group.

[b] P <0.05 versus DM group.

[c] P <0.05 versus DM+AX, 0.01% group.

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