

# Marine collagen peptides reduce endothelial cell injury in diabetic rats by inhibiting apoptosis and the expression of coupling factor 6 and microparticles

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Received June 27, 2016; Accepted April 20, 2017

DOI: 10.3892/mmr.2017.7061

**Abstract.** The present study aimed to elucidate the role of marine collagen peptides (MCPs) in protection of carotid artery vascular endothelial cells (CAVECs) in type 2 diabetes mellitus (T2DM), and the mechanism underlying this process. In an *in vivo* experiment, diabetic Wistar rats were divided randomly into four groups (n=10/group): Diabetes control, and three diabetes groups administered low, medium and high doses of MCPs (2.25, 4.5 and 9.0 g/kg body weight/day, respectively). Another 10 healthy rats served as the control. In an *in vitro* experiment, human umbilical-vein endothelial cells (HUVECs) were incubated in normal and high concentrations of glucose with or without MCPs (3.0, 15.0 and 30.0 mg/ml, respectively) for 24, 48 or 72 h. Blood vessel/endothelial construction, inflammatory exudation and associated molecular biomarkers in CAVECs were detected

and analyzed. The results of the present study demonstrated that in rats, MCP treatment for 4 weeks significantly lowered blood glucose and attenuated endothelial thinning and inflammatory exudation in carotid-artery vascular endothelial cells. *In vitro*, the high-glucose intervention significantly increased cell apoptosis in HUVECs, and medium and high doses of MCPs (4.5 and 9.0 g/kg body weight/day, respectively) partially ameliorated this high glucose-mediated apoptosis and decreased levels of apoptosis biomarkers. In conclusion, a moderate oral MCP dose ( $\geq 4.5$  g/kg body weight/day) may be a novel therapeutic tool to protect against early cardiovascular complications associated with T2DM by inhibiting apoptosis and reducing the expression of coupling factor 6 and microparticles.

## Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disease. Nearly 422 million adults worldwide had diabetes in 2014, compared with 108 million in 1980. The global (age-standardized) prevalence of diabetes in the adult population has nearly doubled since 1980, from 4.7 to 8.5% (1). Diabetes caused 1.5 million mortalities in 2012. Higher-than-optimal blood glucose levels contributed to an additional 2.2 million deaths by increasing the risks of cardiovascular and other diseases (2). Cardiovascular disease (CVD) is a common and serious complication of T2DM, and is linked to increased mortality (3,4). The underlying mechanisms of vascular complications observed in patients with T2DM, as a result from complex interactions between metabolic, inflammatory, and genetic causes and atherothrombotic disease through diverse mechanisms, remain to be fully elucidated (5,6).

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**Key words:** marine collagen peptide, coupling factor 6, microparticles, cell apoptosis, type 2 diabetes mellitus, cardiovascular complications

Therefore, the introduction of novel therapies to reduce the side effects of drugs or minimize the potential harm to arterial vascular endothelial cells induced by high glucose and blood lipid levels would be of great significance.

Bioactive peptides from marine sources form an attractive and promising pool of drug candidates. Marine collagen peptides (MCPs) have been reported to inhibit the activity of angiotensin I-converting enzyme (7), and to demonstrate antihypertensive and hyperlipidemic bioactivity (8). Our previous studies have demonstrated that MCP treatment can inhibit inflammation and oxidative stress and protect  $\beta$ -cells in pancreatic islets from apoptosis in rats with T2DM induced by a high-fat diet (9-11). We also revealed that MCP supplements may have beneficial effects on glucose and lipid metabolism, insulin sensitivity, renal function and hypertension management in Chinese patients with T2DM and hypertension (12-15). However, the underlying mechanisms remain poorly understood.

More recently, links have been established between the expression of coupling factor 6 (CF6) and microparticles (MPs) in hypertension, DM and coronary heart disease. CF6 is released from vascular endothelial cells and binds to the  $\beta$  subunit of the plasma membrane-bound adenosine triphosphate (ATP) synthase in these cells, leading to intracellular acidosis (16). CF6 produces vasoconstriction, and its biologically active site is located in the C-terminal portion. CF6 also suppresses prostacyclin generation via the inhibition of cytosolic phospholipase A2, and inhibits nitric oxide synthase activity via an increase in asymmetrical dimethylarginine and a decrease in platelet/endothelial cell adhesion molecule-1. Thus, CF6 likely contributes to the pathogenesis of CVD (17).

MPs are present in the circulation of healthy individuals. They are cell membrane-derived particles that can stimulate coagulation, inflammation and angiogenesis, and are involved in cell-to-cell communication (18,19). MPs are released from various cell types following inflammation activation or apoptosis. Elevated circulating MP levels are found in patients with various diseases, including acute coronary syndromes, peripheral arterial disease, systemic inflammatory states and T2DM (18,20).

Our previous experiments indicated that a short-term MCP intervention improved cardiac systolic function to a certain extent in patients with hypertension (21), but the role of MCPs in protecting against early T2DM-associated damage to cardiovascular endothelial cells remains unknown. The present study aimed to evaluate the role of MCPs in protecting against T2DM-associated injury of cardiovascular endothelial cells *in vivo* and *in vitro*, and the mechanism underlying this process.

## Materials and methods

**Preparation and identification of MCPs.** MCPs were derived from the skin of wild-caught chum salmon [average body weight (BW) 1.47 kg], which were donated by CF Haishi Biotechnology Ltd. Co. (Beijing, China). The MCPs were prepared and identified according to a method described previously (12,22,23). In brief, the skin of chum salmon was first homogenized and emulsified in distilled water. Second, at 40°C and pH 8, complex protease (Alcalase® 2.4 L,

Protamex®, Novozymes, Bagsvaerd, Denmark) was added for 3 h before inactivation and sterile filtration. Lastly, the MCP mixture was powdered by spray drying. High-performance liquid chromatography (Waters Corporation, Milford, MA, USA), LDI-1700 matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and H835-50 automatic amino acid analysis (Hitachi, Ltd., Tokyo, Japan) were used to determine the molecular weight distribution and amino acid composition. In addition, MCPs were demonstrated to contain very little or no carbohydrate by negative staining of the polyacrylamide gel with periodic acid-Schiff reagent.

**Animal groups and treatments.** Adult male Wistar rats (weight, 160-180 g; age, 6 weeks; n=60) obtained from the Department of Animal Service of Shandong University Health Science Center (Shandong, China) were used for the experiment. The protocol followed the Institutional and National Guidelines for the Care and use of Animals, and the Animal Ethics Review Committee of Southern Medical University Health Science Center (Shenzhen, China) approved all experimental procedures involving animals for this study. The rats were housed individually in rooms with controlled environmental conditions (12-h light/dark cycle, temperature  $\sim 25\pm 2^\circ\text{C}$ , 50% humidity) in an approved animal facility, after a 1-week adaptation period with free access to tap water and standard chow diet (SCD). Rats were assigned randomly to two groups: Normal control (N) group (n=10) and the high-cholesterol, high-fat diet (HCHFD) -induced diabetes model group (n=50).

**Experimental design and T2DM rat model.** T2DM was induced according to methods described in our previous study (18). After 2 weeks of HCHFD feeding, freshly prepared, intraperitoneally-injected streptozotocin (STZ, 30 mg/kg BW) in citrate buffer (0.1 M, pH 4.5) was administered 3 times a week. At 1-2 weeks later, fasting blood glucose (FBG) of rats were detected by a blood glucose meter, and 45 rats with FBG levels  $\geq 11.1$  mmol/l were considered as T2DM and were used for the experiment. A total of 40 of these diabetic rats were then assigned randomly to 4 groups treated with vehicle (group D), or 2.25 (low dose, group L), 4.5 (medium dose, group M) or 9.0 (high dose, group H) g/kg BW/day MCPs (n=10/group) (24). The MCPs and vehicle (distilled water; total volume was 1 ml/application) were administered intragastrically every morning after the T2DM model had been established for 4 weeks.

The same amount of drinking water served as the blank control (group N, n=10). All food and water intake was measured daily, and BW was measured weekly throughout the study.

The composition of the HCHFD (g/100 g food) was: 31% beef tallow, 25% cornstarch, 15.4% casein (78% protein), 8.3% maltodextrin, 6.3% sucrose, 5.0% cellulose, 4% corn oil, 3.5% mineral premix, 1% vitamin premix, 0.3% L-cysteine and 0.2% choline bitartrate. Sucrose solution (30%) was provided as drinking fluid to HCHFD-fed rats. The SCD contained 47% cornstarch, 14.2% maltodextrin, 7% corn oil, no beef tallow, and the same percentages of other ingredients as the HCHFD. SCD-fed rats were provided normal drinking water. Mineral and vitamin mixtures purchased from Harlan Teklad

(Madison, WI, USA) were fed to all rats (25). Rats receiving and not receiving STZ continued on their original diets for the duration of the study.

At days 0 and 28 after treatment, blood samples were collected by retro-orbital sinus puncture under chloroform anesthesia (80-95% chloroform gas; flow rate, 20 ml/min; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). FBG levels were determined using a glucometer. The flow chart of the study is presented in Fig. 1.

**Isolation, culture and treatment of human umbilical-vein endothelial cells (HUVECs).** HUVECs (cat. no. #8000; ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) were collected as described previously (26). They were cultured in HuMedia supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 10 ng/ml recombinant epidermal growth factor, 1 ng/ml hydrocortisone, 5 ng/ml recombinant fibroblast growth factor and 10 ng/ml heparin under 5% CO<sub>2</sub> at 37°C. HUVECs were incubated in a normal glucose concentration (1,000 mg/l, group N), or high glucose concentration (4,000 mg/l, group D) without or with MCPs [3.0 mg/ml (group L), 15.0 mg/ml (group M) or 30.0 mg/ml (group H)] for 24, 48 or 72 h. The dose of MCPs in group H was calculated based on the following formula: typical clinical dose (2.25 g/kg) x standard adult male BW (60 kg)/average blood volume (4,500 ml). The doses in groups M and L were one-fifth and one-tenth those in group H, respectively. Cholecystokinin-octapeptide assays were performed to assess cell growth inhibition using a cell counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), which were detected using an ELISA microplate reader (LabSystems Multiskan MS 352; Thermo Fisher Scientific, Inc.), and the growth inhibition ratio was calculated using the formula: Growth inhibition ratio=[normal control optical density (OD) values-treatment group OD values]/normal control OD values x100%.

**Histological analysis.** Histological analysis was performed as described previously (27). At 4 weeks after HCHFD treatment and MCP interventions, all rats were sacrificed following anesthesia with Fentanyl (0.4 mg/kg; Sigma-Aldrich; Merck KGaA) and carotid artery vascular endothelial cells (CAVECs) were collected. CAVECs were fixed in 4% phosphate-buffered saline-buffered formalin for >24 h, followed by processing for conventional paraffin embedding. Sections (5-um thickness) were mounted on glass slides, dewaxed, rehydrated with distilled water and stained with hematoxylin and eosin. The CAVECs were examined using a light microscope to identify pathological changes.

**Biochemical analysis.** After 28 days of treatment and before sacrifice for organ collection, the animals were fasted overnight and weighed, and blood samples were then collected by heart puncture using a needle under chloroform anesthesia (80-95% chloroform gas; flow rate, 20 ml/min; Sigma-Aldrich; Merck KGaA). Two different tubes with and without anticoagulant (heparin) were used for blood collection in each rat to obtain serum and plasma. Following this, serum and plasma were prepared by centrifuging the blood samples at 1,200 x g and 4°C for 10 min. Plasma glucose levels were estimated using

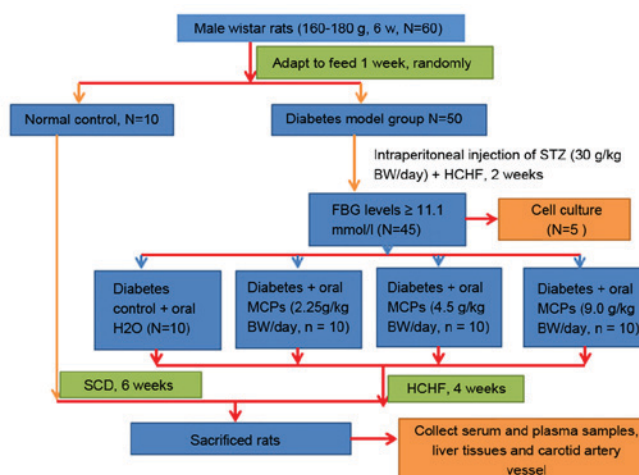


Figure 1. The protocol of present study. BW, body weight; SCD, standard chow diet; HCHF, high-cholesterol high-fat; FBG, fasting blood glucose; MCPs, marine collagen particles; STZ, streptozotocin.

a glucose oxidase activity assay kit (Sigma-Aldrich; Merck KGaA), with a semi-automated analyzer (Photometer 5010 V5+; RIELE GmbH & Co KG, Berlin, Germany).

**ELISA.** Using ELISA kits, the levels of biomarkers including CF6, epithelial membrane proteins (EMPs), peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ; cat. no. CK-CK-E30647H), factor related apoptosis (Fas; cat. no. CK-E076217H), soluble factor-related apoptosis ligand (sFASL; cat. no. CK-E076218H) and MPs (cat. no. CK-E39822R) in plasma, CAVEC and HUVEC culture supernatants were measured using ELISA kits according to the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN, USA) as described previously (28,29). All samples and standards were evaluated in triplicate to ensure the accuracy of results.

**Flow cytometric analysis of HUVEC apoptosis biomarkers with Annexin V-fluorescein isothiocyanate (FITC).** Cells were labeled with Annexin V-FITC (IQP Products BV, Groningen, The Netherlands) and propidium iodide (PI; donated by Dr. E. Reits, Department of Cell Biology and Histology, Academic Medical Centre, Amsterdam-Zuidoost, The Netherlands). Samples were analyzed and cell numbers per culture flask were estimated using flow cytometry (Beckman Coulter Epics XL; Beckman Coulter, Inc., Brea, CA, USA) and Cell Quest software v4.7.0 (BD Biosciences, Franklin Lakes, NJ, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from HUVECs using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-qPCR was performed according to the protocol supplied with the TaqMan Gold RT-PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Oligonucleotide primers and Taq-Man probes for CF6, EMPs, caspase-3, caspase-8 and Fas were designed using Primer Express version 1.5 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The final reaction volume was 25  $\mu$ l and the thermocycling conditions for qPCR were as follows: initial



Table I. Fasting blood glucose values before and after MCP interventions in rats with T2DM induced by a high-carbohydrate, high-fat diet.

Group (n=10 each)	Baseline FBG (tail blood, mmol/l)	P <sup>a</sup>	Post-intervention FBG (heart blood, mmol/l)	P <sup>a</sup>
N	6.27±0.98	-	5.64±0.51	-
D	25.00±3.84	<0.0001	24.70±4.30	<0.0001
L	23.42±3.65	<0.0001	24.56±2.85	<0.0001
M	22.80±3.05	<0.0001	21.00±4.53	<0.0001 <sup>b</sup>
H	22.33±3.10	<0.0001	20.97±1.69	<0.0001 <sup>c</sup>

Data are presented as the mean ± standard deviation. <sup>a</sup>t-test.; <sup>b</sup>P=0.021, M vs. D. <sup>c</sup>P=0.013, H vs. D. FBG, fasting blood glucose; MCP, marine collagen peptide; T2DM, type II diabetes mellitus; N, control group; D, diabetes model group, treated with vehicle only; L, low-dose group; M, medium-dose group; H, high-dose group.

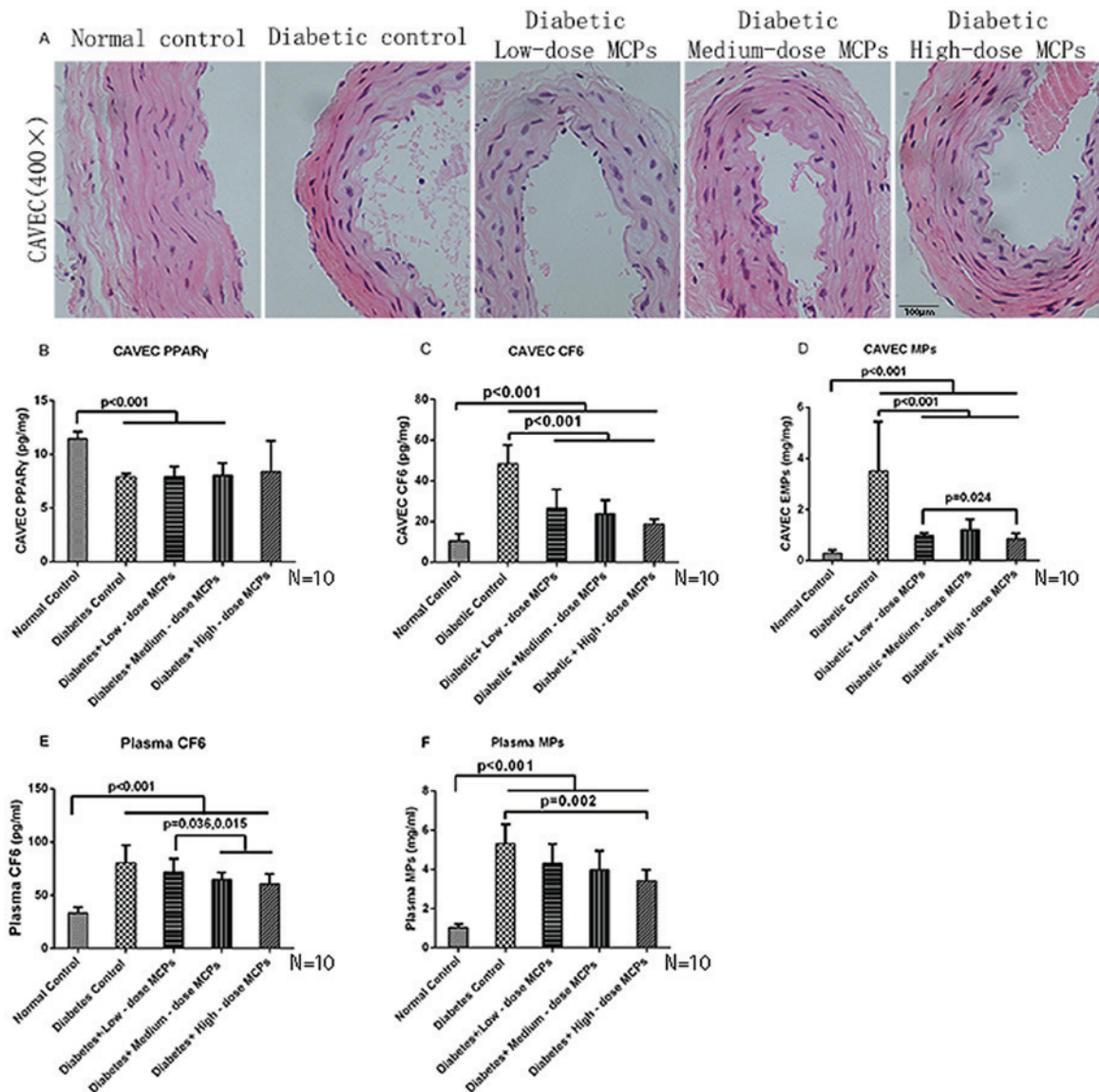


Figure 2. (A) Histological characteristics of CAVECs in the five groups after MCP intervention, as assessed by hematoxylin and eosin staining (magnification, x400). Levels of (B) PPAR $\gamma$ , (C) CF6 and (D) MP in CAVECs, and levels of (E) CF6 and (F) MPs in plasma. Data are presented as the mean ± standard deviation. CAVECs, carotid artery vascular endothelial cells; CF6, coupling factor 6; MP, microparticles; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; MCPs, marine collagen peptides.

Table II. Coupling factor 6 and microparticles expression in plasma and carotid artery vascular endothelial cells in rats after MCP or vehicle intervention<sup>a</sup>.

Tissue	Biomarker	N	D	L	M	H
Plasma	CF6 (pg/ml)	37.29±5.46	74.25±14.10 <sup>b</sup>	75.54±9.07 <sup>b</sup>	67.45±6.62 <sup>b</sup>	65.74±7.08 <sup>b,c</sup>
	MPs (mg/ml)	1.32±0.44	4.66±0.84 <sup>b</sup>	4.15±0.82 <sup>b</sup>	3.98±0.74 <sup>b</sup>	3.51±0.52 <sup>b,d</sup>
CAVEC	CF6 (pg/mg)	10.32±2.59	47.86±4.8 <sup>b</sup>	32.27±4.5 <sup>b,e</sup>	28.78±3.20 <sup>b,e</sup>	30.91±5.21 <sup>b,e</sup>
	MPs (mg/mg)	0.27±0.08	2.46±0.55 <sup>b</sup>	1.56±0.37 <sup>b,e</sup>	1.52±0.51 <sup>b,e</sup>	1.15±0.37 <sup>b,e,f</sup>
	PPAR $\gamma$ (pg/mg)	11.45±0.67	7.8±0.29 <sup>b</sup>	7.93±0.93 <sup>b</sup>	8.04±1.13 <sup>b</sup>	8.36±2.91

Data are presented as the mean  $\pm$  standard deviation. <sup>a</sup>t-test; <sup>b</sup>P<0.001 vs. N; <sup>c</sup>P=0.036 vs. D, P=0.015 vs. L; <sup>d</sup>P=0.002 vs. D; <sup>e</sup>P=0.001 vs. D; <sup>f</sup>P=0.024 vs. L. MCP, marine collagen peptide; N, control group; D, diabetes model group, treated with vehicle only; L, low-dose group; M, medium-dose group; H, high-dose group; MPs, microparticles; CF6, coupling factor 6; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; CAVEC, carotid artery vascular endothelial cell.

denaturation at 94°C for 3 min, then 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and a final extension of 72°C for 10 min. The forward and reverse primers for qPCR were as follows:  $\beta$ -actin, forward, GTGGGGCGCCCCAGGCAGGCA CCA and reverse, CTCCTTAATGTCACGCACGATTTTC (540 bp); Fas, forward, GGACCCAGAATACCAAGTGC and reverse, GCCACTGTTTCAGGATTTAAGG (238 bp); sFasL, forward, CCTTGGTAGGATTGGGCCTG and reverse, GTT GCAAGATTGACCCCGGA (304 bp); caspase-8, forward, GGACAGGAATGGAACACACTT and reverse, TCAGGA TGGTGAGAATATCATC (557 bp); caspase-3, forward, TTT TTCAGAGGGGATCGTTG and reverse, TCAAGCTTGTCG GCATACTG (296 bp; Biosune); PPAR $\gamma$ , forward, CACCTC GGTCTCCCCAGA and reverse, TCAATTGCCATGAGG GAGTTGGAA, (529 bp); CF6 forward, GCCCAATCCGC TTTGTTTT and reverse, CACCACCTCCGCTCTACTTC (408 bp); and EMPs, forward, CACCGGAGGGGACTTAT GG, and reverse, CGCACACAGAGGGTGTGTAT (200 bp). Values were averaged from duplicate data and normalized with human  $\beta$ -actin by the 2<sup>- $\Delta\Delta C_q$</sup>  method (30).

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation, and the significance of differences between experimental and control values were examined using one-way analysis of variance and a least significant difference post-hoc test for multiple comparisons. SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used to perform analyses. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effects of diets on blood glucose levels.** FBG levels were significantly higher in HCHFD-fed rats (groups D, L, M, and H) compared with SCD-fed rats (group N; all P<0.05; Table I). Baseline FBG levels were significantly higher in groups D, L, M and H than in group N. After 4 weeks of MCP interventions, FBG levels were significantly lower in groups M and H than in group D (21.00±4.53 and 20.97±1.69 vs. 24.70±4.30 mmol/l; P=0.021 and 0.013, respectively; Table I).

**Effects of MCPs on CAVECs, CF6, MPs and PPAR $\gamma$  in vivo.** CAVECs from rats in groups D, L, M and H exhibited different

degrees of endothelial thinning and inflammatory exudation. MCP interventions partly inhibited the inflammatory response in CAVECs; this effect was most pronounced in group H (Fig. 2A).

Furthermore, PPAR $\gamma$  levels in CAVECs were significantly reduced in groups D, L and M compared with group N (all P<0.001). PPAR $\gamma$  levels were reduced in group H compared with group N, but this difference was not significant, suggesting that high-dose MCPs improved the expression of PPAR $\gamma$  in CAVECs (Fig. 2B).

ELISA data demonstrated that CF6 levels in plasma and CAVECs were significantly increased in groups D, L, M and H than in group N (P=0.001). Furthermore, CF6 levels in plasma were significantly reduced in group H compared with groups D and L (P=0.036 and 0.015, respectively). Notably, CF6 levels in CAVECs were significantly reduced in groups L, M and H than in group D (all P<0.001), but no significant difference among these three groups was detected. Thus, a certain MCP dosage appeared to improve the diastolic function of endothelial cells (Fig. 2C; Table II).

Similarly, MP levels in plasma and CAVECs were significantly higher in groups D, L, M and H than in group N (all P<0.001). MP levels in plasma were significantly reduced in group H than in group D (P=0.002). MP levels in CAVECs were significantly lower in groups L, M and H compared with group D (all P<0.001). MP levels were significantly reduced in group H compared with group L (P=0.024; Fig. 2D; Table II).

**Effects of MCPs on HUVEC growth in vitro.** In *in vitro* experiments, HUVECs cultured with a high glucose concentration demonstrated increased cell growth inhibition, particularly with prolonged culture (72 vs. 24 h, P<0.01; Fig. 3, Table III). At 24 h of culture, MCP treatment effectively reversed the inhibition of cell growth (groups M and H vs. group D, P<0.05). The protective effect of MCPs on HUVECs was also observed at 72 h after MCP intervention (groups L, M and H vs. group D, P<0.05; Fig. 3; Table III).

**Effects of MCPs on CF6, MPs and PPAR $\gamma$  in the supernatant of HUVEC cultures.** *In vitro* experiments demonstrated that HUVEC CF6 (Fig. 4A) and MP (Fig. 4B) levels in supernatant were elevated after 48 and 72 h culture with a high glucose

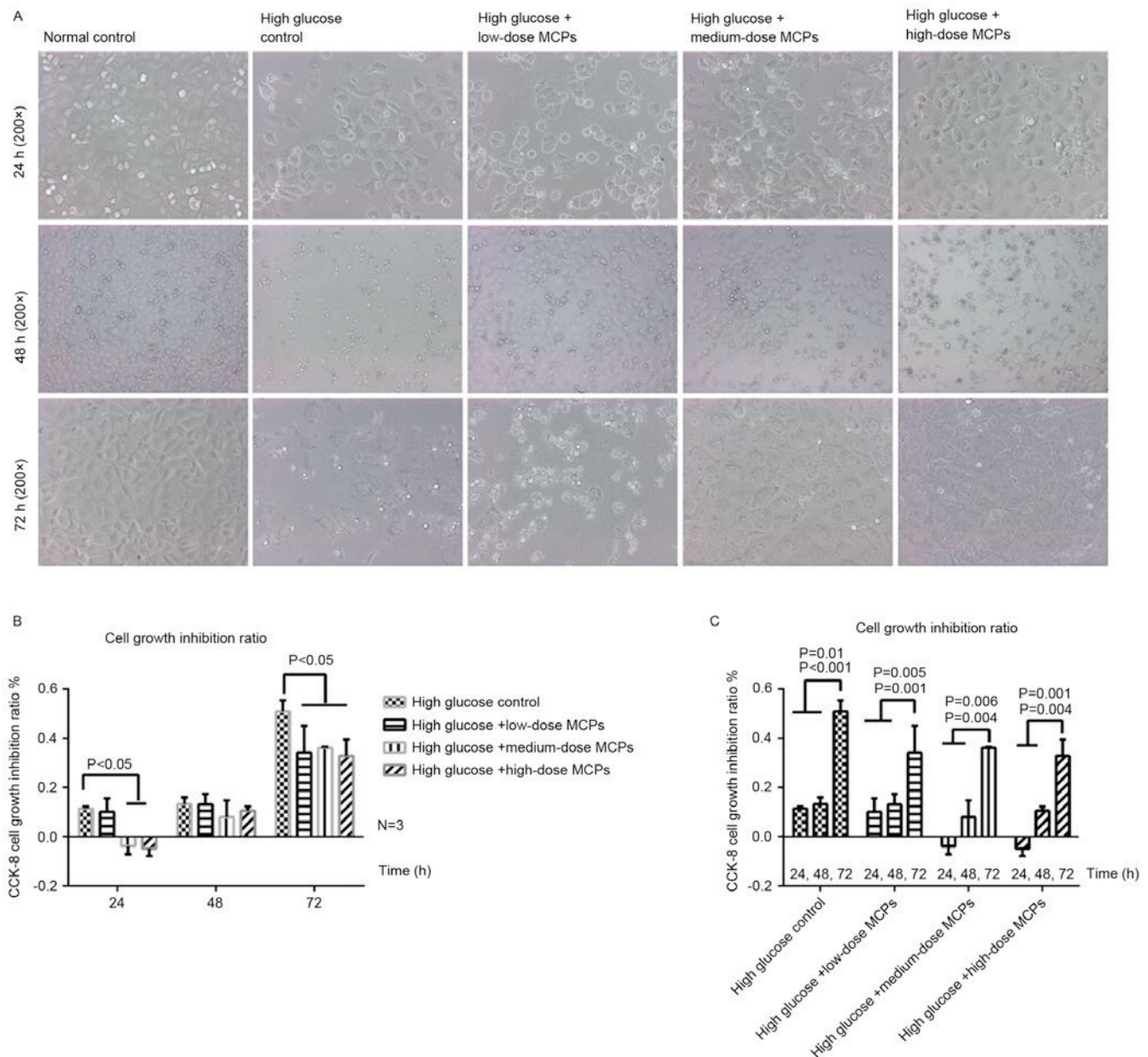


Figure 3. Effects of MCPs on HUVEC growth after 24, 48 and 72 h. (A) HUVECs cultured with high glucose displayed increased cell growth inhibition, which was effectively reversed by MCP treatment. (B) Inter-group comparison of cell growth inhibition ratios at 24, 48, and 72 h. (C) Comparison of cell growth inhibition ratios among time points. Data are presented as the mean  $\pm$  standard deviation. HUVECs, human umbilical-vein endothelial cells; CCK-8, Cell Counting kit-8; MCPs, marine collagen peptides.

concentration. MCP treatment significantly downregulated these elevated CF6 and MP levels at 48 and 72 h ( $P < 0.05$  and  $P < 0.01$ , respectively), particularly in group H, where CF6 levels were comparable to those of group. The opposite trend was observed for PPAR $\gamma$  levels: MCP treatment significantly increased PPAR $\gamma$  in the supernatant of HUVEC culture in groups M and H at 72 h compared with the other groups ( $P < 0.05$ ; Fig. 4C; Table III).

**Effects of MCPs on the expression of apoptosis biomarkers caspase-3, caspase-8 and Fas in HUVECs, as detected by RT-qPCR.** After a 72-h cell culture, MCPs significantly decreased the expression of caspase-3 in HUVECs cultured with a high-glucose concentration, especially in groups M and H (Fig. 5A). High glucose decreased the level of caspase-8 in

comparison with the control, and MCP treatment ameliorated this effect (Fig. 5B). Fas expression levels had a similar pattern to caspase-3 (Fig. 5C).

**Effects of MCPs on the expression of apoptosis biomarkers Fas, sFasL and Annexin-V in HUVECs, as detected by ELISA or flow cytometry.** The expression of Fas and sFasL increased when exposed to high glucose (group H vs. group N,  $P < 0.05$ ; Fig. 6A and B, respectively), and this expression increased continuously with prolonged culture (72 vs. 24 h,  $P < 0.05$ ). Interestingly, MCPs partially restored the expression of Fas and sFasL at 48 and 72 h (MCP treatment groups vs. group D,  $P < 0.05$ ), although this expression remained higher than that observed in group N. Similarly, a high dose of MCPs (30.0 mg/ml) significantly decreased the high glucose-induced



Table III. Peroxisome proliferator-activated receptor  $\gamma$  and apoptosis-associated biomarkers of incubated human umbilical-vein endothelial cells, detected by ELISA or flow cytometry<sup>a</sup>.

Biomarker	Incubation (h)	Normal control	High glucose control	High glucose + low MCP dose	High glucose + medium MCP dose	High glucose + high MCP dose
Growth inhibition ratio (%)	24	1.0	0.114±0.014	0.101±0.095	-0.038±0.058 <sup>c</sup>	-0.048±0.053 <sup>c</sup>
CF6 (pg/ml)	48	1.0	0.132±0.048	0.131±0.073	0.080±0.117	0.106±0.029
	72	1.0	0.510±0.076 <sup>ff,gg</sup>	0.342±0.189 <sup>ff,gg</sup>	0.361±0.007 <sup>ff,gg</sup>	0.395±0.137 <sup>ff,gg</sup>
	24	355.05±24.43	373.09±20.78	388.12±33.17	343.64±22.68	295.85±17.43 <sup>c</sup>
	48	375.82±26.62	728.50±18.32 <sup>bb,ff</sup>	676.04±12.89 <sup>bb,ff</sup>	460.02±27.16 <sup>cc,dd,f</sup>	407.92±25.01 <sup>cc,dd,f</sup>
	72	375.51±18.89	721.58±14.42 <sup>bb,ff</sup>	684.58±59.91 <sup>bb,f</sup>	512.64±31.50 <sup>bb,cc,ff</sup>	484.86±32.73 <sup>bb,cc,ff</sup>
EMPs (mg/ml)	24	13.52±1.01	124.52±2.25 <sup>bb</sup>	109.46±2.75 <sup>bb,cc</sup>	116.27±5.11 <sup>bb</sup>	106.06±4.83 <sup>bb,c</sup>
	48	16.04±1.05	130.89±2.14 <sup>bb</sup>	97.53±1.05 <sup>bb,cc,f</sup>	104.29±2.36 <sup>bb,cc</sup>	93.51±2.12 <sup>bb,cc,e</sup>
	72	15.03±1.54	143.97±2.21 <sup>bb,ff,gg</sup>	107.75±0.67 <sup>bb,cc,gg</sup>	108.37±4.73 <sup>bb,cc</sup>	99.18±1.27 <sup>bb,cc,d</sup>
	24	147.90±18.79	166.05±26.58	150.58±22.24	139.16±23.82	147.75±16.06
	48	140.07±14.94	126.11±24.03	137.90±18.49	137.09±19.67	159.69±21.31
Fas (ng/ml)	72	158.86±12.82	123.65±12.72	143.31±28.27	250.27±10.96 <sup>bb,cc,ff,gg</sup>	243.40±8.72 <sup>bb,cc,d,ff,gg</sup>
	24	28.19±1.51	29.32±1.66	29.07±1.88	27.30±2.47	29.09±2.32
	48	34.01±1.11 <sup>f</sup>	72.93±1.84 <sup>bb,ff</sup>	58.53±1.01 <sup>bb,cc,ff</sup>	52.06±3.02 <sup>bb,cc,ff</sup>	54.20±2.37 <sup>bb,cc,ff</sup>
	72	33.71±1.99	81.97±1.34 <sup>bb,ff,g</sup>	63.84±1.92 <sup>bb,cc,ff</sup>	54.24±1.46 <sup>bb,cc,d,ff</sup>	55.58±2.47 <sup>bb,cc,ff</sup>
	24	60.91±2.71	70.48±2.88	64.81±2.91	58.00±2.39 <sup>c</sup>	61.04±1.46
sFasL (pg/ml)	48	62.69±1.61	75.46±6.84	78.35±7.38	82.98±7.40	71.10±3.87
	72	52.43±2.79 <sup>g</sup>	96.04±4.09 <sup>bb,ff</sup>	79.40±4.10 <sup>bb,c,f</sup>	81.25±8.23	82.81±4.80 <sup>bb,f</sup>
	24	69.47±5.46	83.30±4.36	69.73±10.78	62.17±17.25	33.47±3.15 <sup>bb,cc</sup>
Annexin-V/PI (%)	48	59.27±4.64	81.13±4.70 <sup>b</sup>	58.53±4.57 <sup>c</sup>	67.63±4.74	67.97±3.57
	72	70.63±14.56	79.57±8.33	77.07±2.63	72.47±2.86	67.63±2.38 <sup>c</sup>

Data are presented as the mean ± standard deviation. <sup>a</sup>t-test; <sup>b</sup>P<0.05, <sup>bb</sup>P<0.01 vs. N; <sup>c</sup>P<0.05, <sup>cc</sup>P<0.01 vs. D; <sup>d</sup>P<0.05, <sup>dd</sup>P<0.01 vs. L; <sup>e</sup>P<0.05, <sup>ee</sup>P<0.01 vs. M; <sup>f</sup>P<0.05, <sup>ff</sup>P<0.01 vs. 24 h; <sup>g</sup>P<0.05, <sup>gg</sup>P<0.01 vs. 48 h. MCP, marine collagen peptide; MPs, microparticles; CF6, coupling factor 6; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; EMPs, epithelial membrane proteins; sFasL, soluble factor-related apoptosis ligand; Fas, factor related apoptosis; N, control group; D, diabetes model group, treated with vehicle only; L, low-dose group; M, medium-dose group; H, high-dose group.

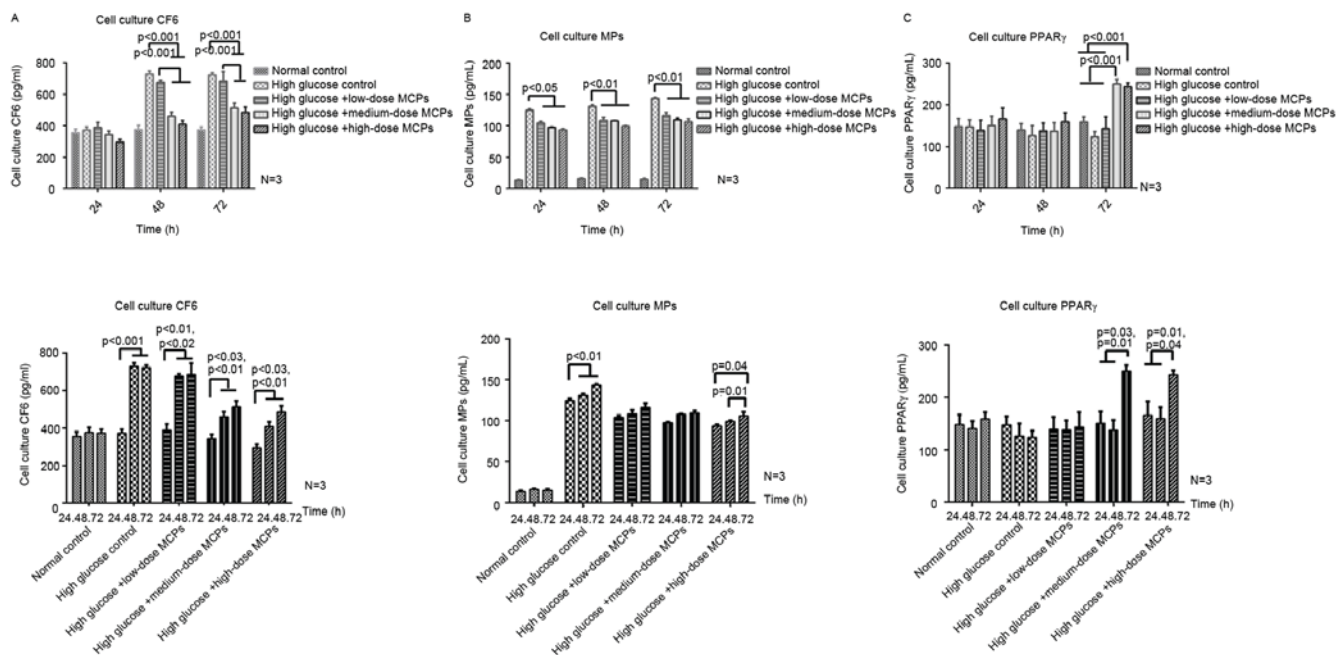


Figure 4. Effects of marine collagen peptides on CF6 and PPAR $\gamma$  in the supernatant of HUVEC cultures, as detected by ELISA. (A) CF6, (B) MP and (C) PPAR $\gamma$  levels in the five groups at 24, 48 or 72 h. Data are presented as the mean  $\pm$  standard deviation. HUVEC, human umbilical-vein endothelial cell; CF6, coupling factor 6; MP, microparticles; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ .

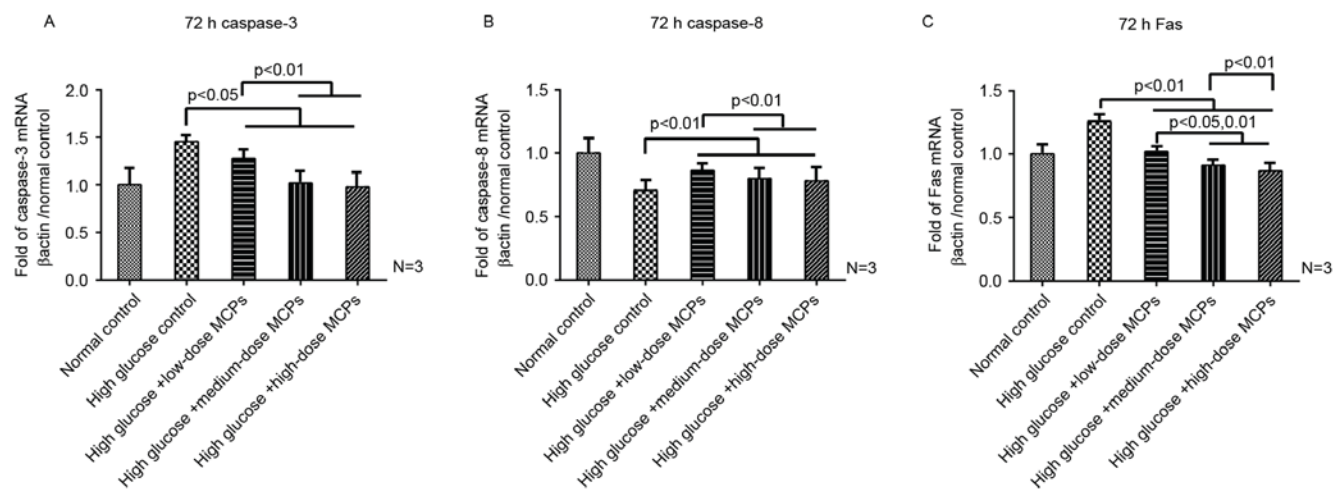


Figure 5. Effects of MCPs on apoptosis biomarkers caspase-3, caspase-8 and Fas in HUVECs. The mRNA expression of (A) caspase-3, (B) caspase-8 and (C) Fas was detected by reverse transcription-quantitative polymerase chain reaction.  $\beta$ -actin served as an internal control. Data are presented as the mean  $\pm$  standard deviation. Fas, factor related apoptosis; MCPs, marine collagen peptides; HUVECs, human umbilical-vein endothelial cells.

elevated expression of Annexin-V/PI in HUVECs (group H vs. group D,  $P < 0.05$ ; Fig. 6C; Table III).

**Discussion**

Our previous research demonstrated that MCPs benefit vasodilatation function in patients with T2DM and hypertension (8,21), and that that oligopeptides from marine salmon skin reduced FBG levels by downregulating T2DM-related oxidative stress and inflammation in diabetic rats (11). However, the molecular mechanisms underlying these beneficial effects of MCPs have not been identified. In the present study, MCP treatment for 4 weeks significantly lowered the blood glucose level

and attenuated endothelial thinning and inflammatory exudation in CAVECs. The present study provides the first evidence that the inhibitory effects of MCPs on CF6 and MPs, as well as apoptosis-associated factors, may contribute to their protection of cardiovascular endothelial cells in a T2DM rat model.

Although T2DM is associated with long-term complications that affect the eyes, kidneys, and peripheral and autonomic nervous systems, CV complications, including hypertension, MI and stroke, are major contributors to mortality in patients with T2DM (31). Intensive control of blood glucose by hypoglycemic agents and/or insulin has been demonstrated to have long-term beneficial effects, reducing the risk of CVD in patients with DM (32). However, the development of novel tools inhibiting



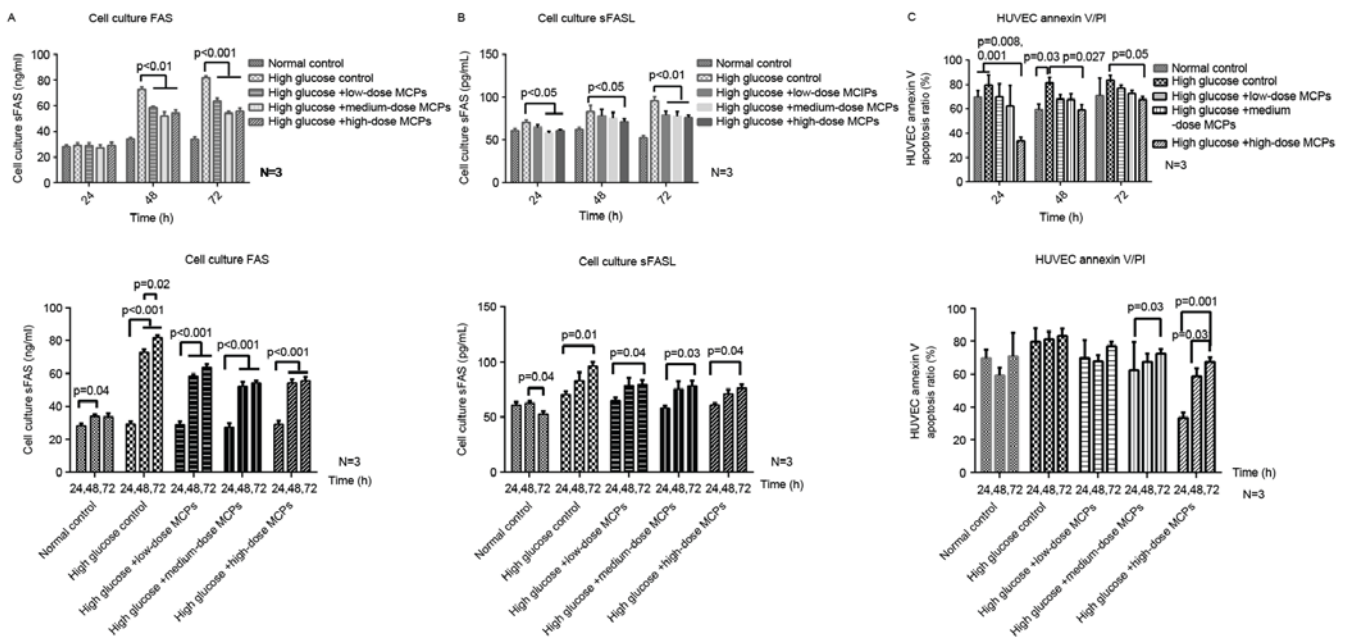


Figure 6. Effects of MCPs on the expression of apoptosis biomarkers in HUVECs, as detected by ELISA or flow cytometry. (A) Fas, (B) sFasL and (C) Annexin-V levels at 24 h, 48 h and 72 h in the five groups. sFasL, soluble factor-related apoptosis ligand; Fas, factor related apoptosis; MCPs, marine collagen peptides; PI, propidium iodide; HUVECs, human umbilical-vein endothelial cells.

the progression in T2DM may be important for the treatment and prevention of diabetic CV complications. The discovery of novel bioactive compounds from marine sources has proceeded at an ever-increasing rate since the first marine compounds were described in the 1980s (14). Compared with synthetic compounds, these natural products from marine organisms have larger-scale structural diversity. Because marine-derived bioactive peptides have numerous health beneficial effects, including anti-hypertensive, -diabetes, -oxidant, -obesity and -aging effects, they have attracted extensive research interest (15). Bioactive peptides derived from marine organisms are known to exhibit a wide range of physiological or hormone-like biological activities that extend beyond their nutritional value (23). For example, eicosapentaenoic acid and docosahexaenoic acid, two major omega-3 fatty acids of marine origin, have been demonstrated to effectively lower blood pressure (33), treat obesity (23) and reduce adipose inflammation (34). Furthermore, their use is recommended in guidelines for the management of patients after myocardial infarction (35). Thus, these observations on MCPs may support novel therapeutic applications for prevention CVD in patients with DM.

The most important finding of the present study was that MCPs exert potent protective actions on cardiovascular tissues, such as endothelial cells, in the T2DM rat model. MCPs are low-molecular-weight peptides extracted from collagen tissues of marine fish, including skin, bone and scales, which account for 30% of marine fish processing waste (36). Previous investigations have confirmed that these protein fragments are safe in animals and humans (24). We previously tested the therapeutic effects of MCPs in diabetic animals (13) and patients (10,12,21). Diabetic rats displayed higher levels of serum tumor necrosis factor- $\alpha$ , interferon- $\gamma$  and malondialdehyde, and lower levels of serum superoxide dismutase and glutathione. Notably, MCP treatment

markedly repressed these detrimental alterations in diabetic rats (13). Furthermore, MCPs significantly reduced blood levels of fasting glucose, fasting insulin, total TGs, TC, LDL-C, glycated hemoglobin A1c and free-fatty acids in patients with T2DM (12). Therefore, we concluded that MCP-induced improvement in metabolic dysfunction helped to retain regular cardiovascular function in T2DM. As expected, it was demonstrated that MCP treatment decreased blood glucose levels and attenuated endothelial thinning and inflammatory exudation in CAVECs in diabetic rats. These results suggested that the attenuation of impaired endothelial function is due to the prevention of high glucose exposure.

Potential molecular mechanisms underlying the protective effect of MCPs were identified in this study. First, it was observed that MCPs depressed the increases in CF6 levels in blood and CAVECs in diabetic rats. CF6 is a component of mitochondrial ATP synthase that induces vasoconstriction. Intravenous injection of CF6 peptide has been demonstrated to increase blood pressure, potentially by suppressing prostacyclin synthesis, whereas a specific neutralizing antibody against CF6 decreased systemic blood pressure concomitantly with an increase in plasma prostacyclin (37). Diabetic patients have been identified to have significantly increased plasma CF6 levels, which are correlated positively with blood glucose and lipid levels (38). CF6 induces insulin resistance, mild glucose intolerance and elevated blood pressure in mice by binding to the plasma membrane ATP synthase [subunit of ecto-F(1)F(0) complex] (34). MPs are released from platelets, leukocytes and endothelia during their activation or death (39). Circulating MPs can penetrate cells, transfer antigens, affect cell gene expression and activate signaling pathways associated with cell proliferation, survival, adhesion and chemotaxis (40,41). Diabetic patients exhibit higher blood levels of MPs (9). MPs isolated from diabetic rats, but not those isolated from control rats, impair the

endothelium-dependent relaxation induced by acetylcholine in rat carotid arteries by downregulating endothelial nitric oxide synthase and overexpressing caveolin-1 (10). Considering these detrimental effects of CF6 and MPs, it was hypothesized that the inhibitory effects of MCPs on local and blood CF6 and MP levels are among the mechanisms underlying the beneficial effects of MCPs; namely, the early protection of cardiovascular endothelial cells and further prevention of CVD in patients with T2DM. Further molecular studies are required to investigate the protective action of MCPs. In addition, biomarkers and techniques for the early diagnosis of cardiovascular endothelial cell injury in T2DM are urgently required, as the complex nature of the disease renders early detection difficult. The expression levels of CF6 and MPs are highly sensitive for the diagnosis of early injury of cardiovascular endothelial cells in T2DM (42).

High glucose can induce reactive oxygen species and apoptosis in vascular endothelial cells, which may contribute to the development of vascular complications in T2DM. Intervention with HUVECs and high glucose concentrations significantly increases levels of apoptosis-associated proteins, such as caspase-3. In the present study, medium and high doses of MCPs partially inhibited high glucose-mediated endothelial cell apoptosis, as evidenced by the decreased levels of caspase-3, caspase-8, MPs, Fas, sFASL and Annexin-V staining *in vitro*, which may be attributed to the inhibition of p38/c-Jun N-terminal kinase and Janus kinase 2 activation and their natural antioxidative properties (43). Collectively, these findings indicate that certain dose of MCPs are potent cardiovascular protectors in T2DM, revealing that an inhibitory effect of MCPs on apoptosis-associated proteins are likely contributors to this protection. In addition, PPAR $\gamma$  has been reported to improve cardiovascular function and limit atherosclerosis, potentially by modulating lipid metabolism and ameliorating insulin resistance (44,45). The results of the present study demonstrated that MCP treatment significantly increased PPAR $\gamma$  expression in the supernatant of HUVEC culture. The activation of PPAR $\gamma$  is also considered as a contributing factor to this protection.

In conclusion, to the best of our knowledge, the results of the present study provided the first evidence that MCPs protect against cardiovascular endothelial cell injury and alleviate cardiovascular dysfunction in a T2DM rat model by downregulating systemic and local CF6 and MP levels, and reduce cell apoptosis and stimulate the expression and activity of PPAR $\gamma$ . These results supported the notion that a medium or high oral dose of MCPs ( $\geq 4.5$  g/kg BW/day) may be a novel therapeutic tool to protect against early cardiovascular complications associated with T2DM.

### Acknowledgements

The present study was supported by the Science and Technology Research and Development Funds of the Shenzhen Science and Technology Innovation Committee (grant no. JCYJ20130319164732236).

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