Food and Chemical Toxicology 126 (2019) 192–198

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Combination of carnosine and asiatic acid provided greater anti-inflammatory protection for HUVE cells and diabetic mice than individual treatments of carnosine or asiatic acid alone

Sheng-lei Yan[a,1], Zhi-hong Wang[b,1], Mei-chin Mong[b], Ya-chen Yang[b], Mei-chin Yin[b,c,*]

[a] Division of Gastroenterology, Department of Internal Medicine, Chang Bing Show-Chwan Memorial Hospital, Changhua County, Taiwan
[b] Department of Food Nutrition and Health Biotechnology, Asia University, Taichung City, Taiwan
[c] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung City, Taiwan

ARTICLE INFO

Keywords:
Carnosine
Asiatic acid
HUVE cell
Diabetes
Apoptosis
p38MPAK

ABSTRACT

The purpose of present HUVE cells and mice study was to investigate the combined effects of carnosine and asiatic acid (AA) against diabetic progression. In HUVE cells, high glucose decreased cell viability, reduced Bcl-2 mRNA expression and increased Bax mRNA expression. The co-treatment of 0.5 μM carnosine plus 0.5 μM AA led to greater cell viability and Bcl-2 mRNA expression than 1 μM carnosine or 1 μM AA treatment alone. This combination more significantly decreased the production of DNA fragmentation, tumor necrosis factor (TNF)-alpha, reactive oxygen species (ROS), and nuclear factor kappa B binding activity than carnosine or AA treatment alone. In diabetic mice, the combination of 0.25% carnosine plus 0.25% AA in diet resulted in higher final body weight, and lower levels of plasma glucose and triglyceride than 0.5% carnosine or 0.5% AA treatment alone. Carnosine and AA combination caused more reduction in renal levels of leukin-6, TNF-alpha and ROS than carnosine or AA treatment alone. This combination also more significantly limited renal cyclooxygenase-2 activity and p-p38 phosphorylation than carnosine or AA treatment alone. These novel findings support that this combination is a more powerful remedy for diabetic control.

1. Introduction

Carnosine (β-alanyl-L-histidine), a small hydrophilic endogenous dipeptide, abundantly exists in skeletal muscles; and distributes in organs such as brain, kidney and heart (Jackson and Lenney, 1996). People could obtain this peptide by dietary intake of fish or meats (Abe et al., 1993). It is reported that carnosine has preventive, protective and therapeutic effects for cardiac malfunctions, hepatic diseases and diabetes (Kamei et al., 2008; Ozdoğan et al., 2011; Fouad et al., 2017). Those authors indicated that carnosine executes those bio-functions through its anti-inflammatory, anti-glycative and anti-oxidative activities. Asiatic acid (AA), a pentacyclic triterpenic acid, is presented in some medicinal plants such as *Centella asiatica* and *Maytenus procumbens* (Puttarak and Panichayupakaranant, 2012; Momtaz et al., 2013). Animal studies revealed that AA could improve pulmonary fibrosis, cardiac hypertrophy and diabetes via its anti-oxidative, anti-fibrotic, hypolipiemic, hypoglycemic and anti-inflammatory actions (Ramachandran et al., 2014; Si et al., 2014; Dong et al., 2017). Although it is known that carnosine or AA has anti-diabetic potent, less attention is paid to the combined effects of carnosine and AA against diabetes. This idea is from the stronger anti-oxidative activities of carnosine plus vitamin E than either carnosine or vitamin E treatment alone against doxorubicin-induced toxicity in heart and liver of rats (Kumral et al., 2016). That is, the combination of a hydrophilic agent like carnosine and a lipophilic agent like AA may more efficiently prevent or ameliorate diseases. The other possible advantages of these combinations include saving the therapeutic expenditure and diminishing the side effects of using one single agent at higher doses or longer time. For instance, AA is a lipophilic compound, and the intake of this agent at higher doses or longer time might enhance metabolic burden,

Abbreviations: AA, asiatic acid; BW, body weight; COX, cyclooxygenase; DCFH-DA, dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVE, human umbilical vein endothelial; IL, interleukin; MAPK, mitogen-activated protein kinase; MMP, mitochondrial membrane potential; NF-κ, nuclear factor kappa; PGE, prostaglandin E; RFU, relative fluorescence unit; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; TNF, tumor necrosis factor

* Corresponding author. Department of Food Nutrition and Health Biotechnology, Asia University, Taichung City, Taiwan.
E-mail address: mcyin@hotmail.com.tw (M.-c. Yin).

[1] These two authors contributed equally.

https://doi.org/10.1016/j.fct.2019.02.027
Received 20 November 2018; Received in revised form 29 January 2019; Accepted 18 February 2019
Available online 22 February 2019
0278-6915/

especially for subjects with liver diseases.

Apoptotic and inflammatory stresses are responsible for the progression or deterioration of diabetes (Brownlee, 2001). The activation of signaling pathways such as caspases, mitogen-activated protein kinase (MAPK) and nuclear factor kappa (NF-κ)B promotes the generation of down-stream apoptotic, oxidative and inflammatory factors including reactive oxygen species (ROS), interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-alpha. Subsequently, these factors contribute to diabetes induced cell death and organ failure (Hong et al., 2016; Liao et al., 2017; Elsherbiny et al., 2018). Thus, if the combination of carnosine and AA limits the activation of these signaling pathways, and decreases the production of these down-stream oxidative and inflammatory factors than carnosine or AA treatment alone, this combination may provide more efficient anti-diabetic potent.

Human umbilical vein endothelial (HUVE) cell is a vascular endothelial cell, and commonly used as a cell model to evaluate the protective or alleviative effects of natural compound(s) against the development or deterioration of diabetes (Wu et al., 2018). In present study, HUVE cells were pre-treated with carnosine alone, AA alone, or carnosine plus AA. Then, high glucose was used to induce injury. The effects of these three treatments upon cell viability, plasma membrane integration, Bcl-2 and Bax mRNA expression, inflammatory cytokines levels and NF-κB p50/65 binding activity were examined and compared. In addition, diabetic mice were also used to evaluate and compare the dietary effects of carnosine alone, AA alone, or carnosine plus AA against diabetic progression. The impact of these three treatments upon glycemic control, plasma lipid level, renal levels of inflammatory cytokines and ROS, renal cyclooxygenase (COX)-2 activity and MAPK protein expression was determined.

2. Materials and methods

2.1. Materials

Carnosine (97%) and AA (98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HUVE cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Culture medium, plates, antibiotics and other chemicals required for cell culture were purchased from Difco Laboratory (Detroit, MI, USA). Male Balb/cA mice at 3-week old were provided by National Laboratory Animal Center (Taipei City, Taiwan). Asia University animal care and use committee approved this animal study, and the permission number is 107-asia-06.

2.2. Cell culture and treatments

HUVE cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) under a condition of 95% air and 5% CO_2 at 37 °C. This DMEM contained 10% fetal bovine serum, 1.5 g/l $NaHCO_3$, 5.5 mM glucose, 100U/ml streptomycin and 100U/ml penicillin. Culture medium was refreshed every 3 days. Cell number was adjusted by phosphate-buffered saline (PBS, pH 7.2) to 10^5/ml for experiments. Carnosine was directly added in DMEM, AA was first suspended in 0.8% methylcellulose, and further diluted with DMEM. Cells were pre-treated with 1 μM carnosine, 1 μM AA or 0.5 μM carnosine plus 0.5 μM AA. After 24 h incubation at 37 °C, HUVE cells were treated with DMEM containing high glucose (33 mM), and further incubating for another 24 h at 37 °C. Normal groups were HUVE cells without carnosine, AA or high glucose. Control groups were HUVE cells with high glucose, and without carnosine or AA.

2.3. Assays for cell viability

Cells (10^5/ml) suspended in culture medium were mixed with 0.25 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. After a 3-hr incubation at 37 °C and centrifugation, a Bio-Rad microplate reader (Hercules, CA, USA) was applied to quantify the formazan product by recording the absorbance of cell samples at 570 nm and 630 nm, in which 630 nm was a reference wavelength. The viability of cell sample was reported as a percentage of normal groups.

2.4. Analyses of mRNA expression by reverse transcriptase real-time polymerase chain reaction (RT-PCR)

HUVE cells were homogenized with TRIZOL reagents obtained from Invitrogen Life Technology (Carlsbad, CA, USA). Total mRNA was isolated, and its concentration was determined by measuring the absorbance ratio of 260/280 nm. Values at 1.8–2.0 indicated a pure RNA samples. Then, cDNA was synthesized from 5 μg RNA by using a cDNA synthesis kit (Legene Biosciences, San Diego, CA, USA) according to the instructions of manufacturer. Subsequently, the expression of target genes was analyzed by using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) and a SYBR® Premix Ex TaqTM II kit (Takara Biotechnology Ltd., Dalian, China), which led to the presence and participation of SYBR Green, a fluorescent dye. The PCR reaction solution consisted of 2 μl cDNA, 0.5 μl of reverse primers, 0.5 μl of forward primers and 12.5 μl SYBR containing mixture. The primers of target genes were as follow: Bcl-2 (156 bp), forward, 5′-CGT TTG GCA GTG CAA TGG T-3′, reverse, 5′-TTC TTG ATT GAG CGA GCC TT-3’; Bax (156 bp), forward, 5′-TGG CAG CTG ACA TGT TTT CTG AC-3′, reverse, 5′-TCA CCC AAC CAC CCT GGT CTT-3’. The primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene, 140 bp), forward, 5′-AGA GGC AGG GAT GTT CTG-3′, reverse, 5′-GAC TCA TGA CCA CAG TCC ATG C-3’. The conditions for running PCR were denaturation for 3 min at 95 °C, annealing for 10 s at 60 °C, and extension for 20 s at 72 °C. For Bcl-2 or Bax expression, 35 cycles were processed. For GAPDH expression, 28 cycles were performed. For each sample, RT-PCR was processed in triplicate. Standard curves were created to convert the average threshold cycles into copy numbers. Expressed Bcl-2 or Bax mRNA was normalized against GAPDH mRNA expression.

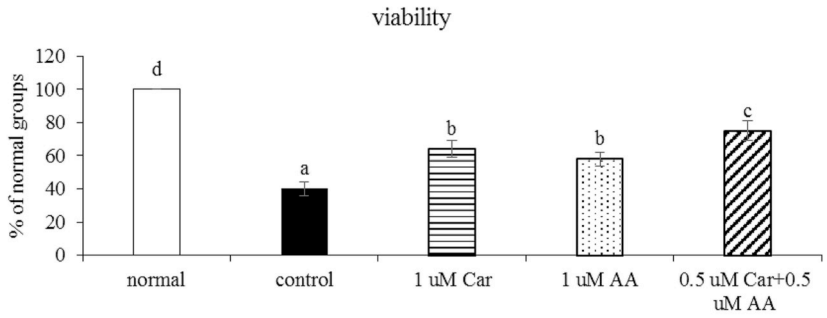
2.5. Measurements for mitochondrial membrane potential (MMP), DNA fragmentation and caspas-3 activity

MMP was measured by using Rh123, a fluorescent dye. Cell samples were treated with Rh123 at 100 μg/l for 30 min at 37 °C. After washed twice with PBS, the mean fluorescence intensity of samples was analyzed by a Beckman-FC500 flow cytometry (Beckman Coulter, Fullerton, CA, USA). MMP was reported as a percentage of normal groups. DNA fragmentation was assayed by using a cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Cell samples were suspended in lysis buffer for 30 min, and followed by centrifugation at 250 × *g* for 10 min. Twenty μl supernatant was mixed with 80 μl immunoreagents, and followed by incubating for 2 h at room temperature. Substrate was added into the mixture, and this mixture was further incubated for 15 min. The absorbance of cell sample at 405 nm and 490 nm was recorded, in which 490 nm was a reference wavelength. DNA fragmentation was reported as a fold of normal groups. Caspase-3 activity was determined by a fluorometric kit (Upstate, Lake Placid, NY, USA). Cell lysates were treated with specific substrates, and followed by incubating for 60 min at 37 °C. Fluorescence value was recorded by a Hitachi F-4500 fluorophotometer (Tokyo, Japan). Emission and excitation wavelengths were set at 505 nm and 400 nm. Caspase-3 activity was reported as folds of normal groups.

2.6. Assays of cytokines and ROS

Cytoscreen kits purchased from BioSource International Co. (Camarillo, CA, USA) were used to analyze the levels of IL-1beta, IL-6 and TNF-alpha in HUVE cells or mice renal homogenates according to the instructions of manufacturers. ROS level was assayed by 2′,7′-

a.



b.

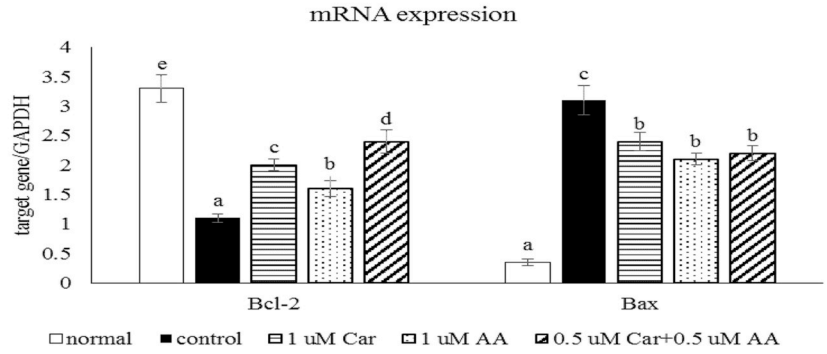


Fig. 1. Effects of Carnosine (Car) and/or AA upon cell viability (a, % of normal groups), and mRNA expression of Bcl-2 and Bax (b, target gene/GAPDH). HUVE cells were pretreated with 1 μM Car, 1 μM AA or 0.5 μM Car + 0.5 μM AA for 24 h incubation at 37 °C. Then, cells were treated with DMEM containing high glucose (33 mM), and followed by incubating for another 24 h at 37 °C. Normal group had no Car, AA, or high glucose. Control group had no Car or AA, but with high glucose. Data are mean ± SD (n = 6). Values with different alphabet among bars are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

dichlorofluorescein diacetate (DCFH-DA). In brief, 100 μl cell lysates or renal homogenate were reacted with 100 μl 2 mg/ml DCFH-DA. A fluorescence plate reader was used to monitor the fluorescence change after 30 min incubation at 37 °C. The excitation and emission wavelengths were respectively set at 488 nm and 525 nm. Result was expressed as relative fluorescence unit (RFU) per mg protein.

2.7. NF-κB p50/65 binding activity assay

Nuclear protein extract of HUVE cells was prepared according to the method of Schilling et al. (1999). Subsequently, this extract at 10 μg was used for measuring NF-κB p50/65 binding activity by an assay kit (Chemicon International Co., Temecula, CA, USA). A Bio-Rad microtiter plate reader (Model 550, Hercules, CA, USA) was applied to monitor the absorbance at 450 nm. Data were expressed as optical density (OD) per mg protein.

2.8. Animal experimental design

Mice with body weight (BW) about 25.1 ± 1.4 g were treated with streptozotocin (40 mg/kg BW) via i.p. injection for continuous 5 days in order to induce type 1 diabetes. Mice with fasting blood glucose levels ≥ 200 mg/dl were considered as diabetic ones, and used for this study. Diabetic mice were further divided into 4 groups, consumed mouse basal diet (control), 0.5% carnosine, 0.5% AA, and 0.25% carnosine plus 0.25% AA. Carnosine was directly supplied in the drink water. AA at 0.5 or 0.25 g was mixed with 99.5 or 99.75 g mouse basal diet. Normal mice without diabetes were used for comparison. After 6-week treatments, mice were sacrificed after overnight fasting. Plasma and kidney were collected.

2.9. Blood analyses

Plasma glucose level (mmol/l) was determined by a glucose HK kit (Sigma Chemical Co., St. Louis, MO, USA). Plasma insulin level (nmol/l) was measured by an assay kit (DRG Instruments Co., Marburg, Germany). Plasma triglyceride and total cholesterol (TC) levels were quantified by assay kits purchased from Wako Pure Chemical Co. (Osaka, Japan). The activities of alanine transaminase (ALT) and aspartate transaminase (AST) in plasma were analyzed by assay kits (Randox Laboratories Ltd., Crumlin, UK). The levels of blood urine nitrogen (BUN) and creatinine (Cr) in plasma were analyzed by using an autoanalyzer (Beckman Coulter, Fullerton, CA, USA).

2.10. Renal analyses

Renal tissue was perfused by PBS for 2 min in order to exclude the left blood. Then, 100 mg renal tissue was mixed with 2 ml PBS, and followed by homogenization with a Teflon glass homogenizer (Glas-Col Co., CA, USA). Renal COX-2 activity and prostaglandin E_2 (PGE_2) level were measured by assay kits (Cayman Chemical Co., Ann Arbor, MI, USA) according to the instructions of manufactures.

2.11. NF-κB p65 and MAPK protein expression in mice kidney

Protein concentration of renal homogenate was determined by Bio-Rad protein assay reagents (Bio-Rad Laboratories Inc. Hercules, CA, USA). Sample with 30 μg protein was used to detect protein expression of NF-κB p65, phosphorylated p38 and phosphorylated JNK by using ELISA kits (Abcam Co., Cambridge, MA, USA) according to manufacturer's instructions.

2.12. Statistical analyses

In cell line study, values were collected from 6 different preparations (n = 6). In animal study, values were obtained from 8 mice (n = 8). Data are reported as mean ± standard deviation (SD). Statistical analyses were handled by one-way analysis of variance.

Table 1
Effects of carnosine (Car) and/or AA upon MMP (% of normal groups), DNA fragmentation (fold of normal groups), and caspase-3 activity (fold of normal groups). HUVE cells were pre-treated with 1 μM Car, 1 μM AA or 0.5 μM Car + 0.5 μM AA for 24 h incubation at 37 °C. Then, cells were treated with DMEM containing high glucose (33 mM), and followed by incubating for another 24 h at 37 °C. Normal group had no Car, AA, or high glucose. Control group had no Car or AA, but with high glucose. Data are mean ± SD (n = 6). Values with different alphabet in a column are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

	MMP % of normal groups	DNA fragmentation fold of normal groups	caspase-3 folds of normal groups
Normal	100^{d}	1.00^{a}	1.00^{a}
Control	33 ± 3^{a}	2.16 ± 0.18^{d}	2.48 ± 0.21^{c}
1 μM Car	68 ± 5^{c}	1.56 ± 0.1^{c}	1.82 ± 0.16^{b}
1 μM AA	57 ± 4^{b}	1.7 ± 0.13^{c}	1.97 ± 0.09^{b}
0.5 μM Car + 0.5 μM AA	60 ± 2^{b}	1.37 ± 0.08^{b}	1.75 ± 0.14^{b}

Dunnett's *t*-test was carried on for Post-hoc comparison. All statistical analyses were processed by using SAS (SAS Institute, Cary, NC, USA). *P* value < 0.05 was considered as significant.

3. Results

3.1. HUVE cell line study

High glucose decreased the viability of HUVE cells (Fig. 1a, $P < 0.05$). High glucose also reduced Bcl-2 mRNA expression and increased Bax mRNA expression (Fig. 1b, $P < 0.05$). The pre-treatments of carnosine or AA at 1 μM reversed these alternations ($P < 0.05$). Furthermore, the co-treatment of 0.5 μM carnosine + 0.5 μM AA led to greater cell viability and Bcl-2 mRNA expression that 1 μM carnosine or 1 μM AA treatment alone ($P < 0.05$). As shown in Table 1, high glucose decreased MMP, and increased DNA fragmentation and caspase-3 activity ($P < 0.05$). Carnosine or AA treatment at 1 μM reversed these changes ($P < 0.05$). The combination of carnosine and AA resulted in more reduction in DNA fragmentation than carnosine or AA treatment alone ($P < 0.05$). High glucose stimulated the release of IL-1beta, IL-6, TNF-alpha and ROS (Table 2, $P < 0.05$). Carnosine or AA treatment at 1 μM lowered the levels of inflammatory cytokines and ROS ($P < 0.05$). The combination of carnosine and AA caused more decrease in TNF-alpha and ROS production than carnosine or AA treatment alone ($P < 0.05$). As shown in Fig. 2, high glucose increased NF-κB binding activity ($P < 0.05$). Carnosine or AA treatment at 1 μM decreased this binding activity ($P < 0.05$). Carnosine and AA combination led to more reduction in NF-κB binding activity ($P < 0.05$).

Table 2
Effects of carnosine (Car) and/or AA upon IL-1beta, IL-6, TNF-alpha level (pg/mg protein) and ROS level (RFU/mg protein). HUVE cells were pre-treated with 1 μM Car, 1 μM AA or 0.5 μM Car + 0.5 μM AA for 24 h incubation at 37 °C. Then, cells were treated with DMEM containing high glucose (33 mM), and followed by incubating for another 24 h at 37 °C. Normal group had no Car, AA, or high glucose. Control group had no Car or AA, but with high glucose. Data are mean ± SD (n = 6). Values with different alphabet in a column are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

	IL-1beta pg/mg protein	IL-6 pg/mg protein	TNF-alpha pg/mg protein	ROS RFU/mg protein
Normal	10 ± 2^{a}	9 ± 3^{a}	12 ± 3^{a}	0.21 ± 0.09^{a}
Control	82 ± 7^{c}	79 ± 5^{c}	94 ± 6^{e}	2.27 ± 0.16^{e}
1 μM Car	53 ± 4^{b}	58 ± 6^{b}	66 ± 5^{d}	1.41 ± 0.12^{c}
1 μM AA	46 ± 5^{b}	50 ± 3^{b}	50 ± 4^{c}	1.68 ± 0.08^{d}
0.5 μM Car + 0.5 μM AA	48 ± 3^{b}	52 ± 4^{b}	39 ± 3^{b}	1.09 ± 0.11^{b}

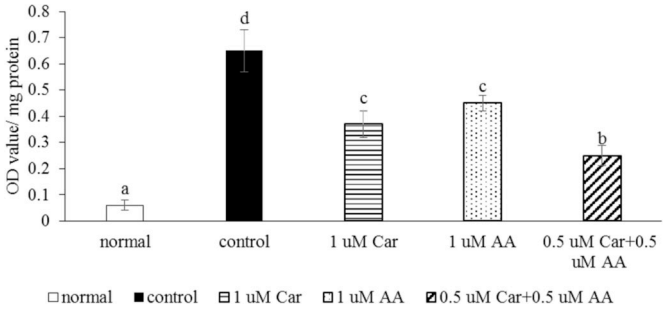


Fig. 2. Effects of carnosine (Car) and/or AA upon NF-κB p50/65 binding activity (OD value/mg protein). HUVE cells were pre-treated with 1 μM Car, 1 μM AA or 0.5 μM Car + 0.5 μM AA for 24 h incubation at 37 °C. Then, cells were treated with DMEM containing high glucose (33 mM), and followed by incubating for another 24 h at 37 °C. Normal group had no Car, AA, or high glucose. Control group had no Car or AA, but with high glucose. Data are mean ± SD (n = 6). Values with different alphabet among bars are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

3.2. Diabetic mice study

As presented in Table 3, diabetes increased water intake and feed intake, but decreased final BW in mice ($P < 0.05$). Carnosine and AA supplementation either alone or combined reduced feed intake and water intake in those diabetic mice ($P < 0.05$). Three supplementations increased final BW ($P < 0.05$), in which the combination of carnosine and AA resulted in higher final BW than carnosine or AA treatment alone ($P < 0.05$). Diabetes and all supplementations did not affect kidney weight ($P > 0.05$). Carnosine or AA supplementation alone for 6 weeks decreased fasting glucose levels and increased insulin levels (Table 4, $P < 0.05$). The combination of carnosine and AA led to lower glucose level than carnosine or AA treatment alone ($P < 0.05$). Carnosine or AA supplementation alone reduced plasma triglyceride content ($P < 0.05$); but failed to affect plasma TC content ($P > 0.05$). The combination of carnosine and AA resulted in more decrease in plasma triglyceride content when compared with carnosine or AA treatment alone ($P < 0.05$). Carnosine and AA supplementation either alone or combined did not affect the activity or level of ALT, AST, BUN and Cr in circulation ($P > 0.05$). Renal levels of cytokines and ROS were shown in Table 5. Carnosine or AA supplementation alone lowered renal levels of these factors ($P < 0.05$). The combination of carnosine and AA caused more reduction in renal levels of IL-6, TNF-alpha and ROS than carnosine or AA treatment alone ($P < 0.05$). Diabetes increased renal COX-2 activity and PGE_2 generation (Fig. 3, $P < 0.05$). Carnosine or AA supplementation alone reversed these alterations ($P < 0.05$). The combination of carnosine and AA more significantly decreased renal COX-2 activity than carnosine or AA treatment alone ($P < 0.05$). As shown in Fig. 4, diabetes up-regulated renal protein expression of NF-κB p65, p-p38 and p-JNK ($P < 0.05$). Carnosine or AA supplementation alone down-regulated the protein expression of these factors ($P < 0.05$). The combination of carnosine and AA led to lower p-p38 expression than carnosine or AA treatment alone ($P < 0.05$).

4. Discussion

The *in vivo* anti-diabetic effects of carnosine or AA have been observed (Kamei et al., 2008; Ramachandran et al., 2014). The data of present study agreed those previous studies, and indicated that either carnosine or AA alone could improve several important pathological features of diabetes. Our present work did not intend to compare the anti-diabetic capabilities between carnosine and AA. We focused on the combined effects of carnosine plus AA against diabetic progression. When compared with carnosine or AA treatment alone, this combination of carnosine plus AA displayed more significant protection for HUVE cells against high glucose induced injury, which were evidenced

Table 3
Water intake (ml/mouse/day), feed intake (g/mouse/day), initial and final body weight (BW, g/mouse), and kidney weight (KW, mg/mouse) in normal mice (N) and diabetic mice without treatment (D), or with 0.5% Car, 0.5% AA or 0.25% Car + 0.25% AA for 6 weeks. Carnosine was added in the drink water. AA at 0.5 or 0.25 g was mixed with 99.5 or 99.75 g mouse basal diet. Values are mean ± SD, n = 8. Values with different alphabet in a column are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

	N	D	0.5 Car	0.5 AA	0.25Car + 0.25AA
WI, ml/mouse/day	2.6 ± 0.8^a	5.9 ± 1.4^c	4.1 ± 0.6^b	4.7 ± 0.4^b	4.2 ± 1.0^b
FI, g/mouse/day	2.4 ± 0.5^a	6.6 ± 1.2^c	4.4 ± 1.0^b	4.2 ± 0.8^b	4.5 ± 0.7^b
Initial BW, g/mouse	24.8 ± 1.2^b	18.4 ± 0.8^a	17.9 ± 0.6^a	18.1 ± 1.0^a	17.7 ± 1.2^a
Final BW, g/mouse	35.1 ± 2.2^d	10.6 ± 1.3^a	13.3 ± 0.7^b	14.5 ± 0.5^b	16.7 ± 0.8^c
KW, mg/mouse	455 ± 17^a	451 ± 12^a	449 ± 14^a	456 ± 8^a	452 ± 11^a

by greater cell survival and Bcl-2 mRNA expression; lower DNA fragmentation and NF-κB binding activity; less TNF-alpha and ROS generation. In addition, our diabetic mice study further revealed that the anti-diabetic effects of this combination was more prominent than carnosine or AA alone. The obtained evidence included higher final body weight, lower plasma glucose and triglyceride levels, less renal IL-6, TNF-alpha and ROS formation, lower renal COX-2 activity and p-p38 expression. These novel findings support that this combination, carnosine plus AA, is a more powerful remedy for diabetic attenuation.

Bcl-2 is an anti-apoptotic factor, and is responsible for regulating cell survival (Schelman et al., 2004). In our present work, carnosine and AA combination resulted in more remarkable Bcl-2 mRNA expression in high glucose treated HUVE cells, which contributed to enhance anti-apoptotic capability and increase HUVE cell viability. DNA fragmentation from high glucose stimulation promoted cell rupture and apoptosis (Kaundal et al., 2006). We found that this combination more effectively stabilized DNA, which consequently benefited cell survival. Higher NF-κB binding activity and over-production of TNF-alpha and ROS facilitate inflammatory and oxidative processes in endothelial cells (Lee et al., 2012). In our cell line study, this combination caused more reduction in NF-κB binding activity, which in turn diminished the production of down-stream inflammatory and oxidative factors such as TNF-alpha and ROS. Since both inflammatory and oxidative stresses have been decreased, it seems reasonable to observe higher viability of HUVE cells under high glucose condition. These results implied that this combination could offer more significant protection at molecular level to enhance the defensive ability of HUVE cells against high glucose induced injury.

In our diabetic animal study, this combination at the used dosages seems safe based on the observed normal values of ALT, AST, BUN and Cr. Our data revealed that this combination more effectively improved glucose and lipid metabolism, and consequently ameliorated hyperglycemia, hyperlipidemia and body weight loss. These improvements definitely benefit healthy management of diabetic subjects (Purnell et al., 1998; Ciudin et al., 2012). In addition, both increased COX-2 activity and activated p38MAPK facilitated inflammatory reactions, and led to massive production of inflammatory cytokines and oxidants under diabetic condition (Adhikary et al., 2004; Bitto et al., 2014). We found this combination caused more reduction in renal COX-2 activity and p38 phosphorylation in diabetic mice. Since these upstream mediators and/or signaling pathways were suppressed, the lower production of IL-6, TNF-alpha and ROS formation in kidney of diabetic mice could be explained. It is reported that oxidative stress stimulates COX-2 activation (Li et al., 2005). Since this combination already mitigated renal oxidative stress through decreasing ROS production, it might be reasonable to find lower renal COX-2 activity. These findings indicated that this combination could markedly suppress COX-2 and p38MAPK pathways, which subsequently diminished inflammatory and oxidative stresses in kidney. Adhikary et al. (2004) indicated that p38 phosphorylation played a crucial role responsible for pathological progression of renal interstitial fibrosis. We found the combination of carnosine plus AA was more powerful in limiting renal p38 phosphorylation; thus, this combination might be useful to prevent or alleviate other renal diseases. These results once again agreed that this combination could provide more efficiently anti-diabetic protection at molecular levels.

Carnosine is a hydrophilic compound and AA is a lipophilic compound. Because of the different chemical properties, it is likely that these two agents via their anti-apoptotic, anti-oxidative and anti-inflammatory actions cooperatively alleviated high glucose (or hyperglycemia) induced toxicity in cellular aqueous and lipidic regions, respectively. Consequently, this combination more significantly stabilized cellular compartments. Carnosine, a peptide, is presented in many muscle foods such as fish and chicken (Peiretti et al., 2011). AA, a triterpenoic acid, is abundant in many vegetables, fruits or herbs such as hawthorn fruit (*Crataegi Pinnatifidae* Fructus), licorice (*Glycyrrhiza glabra*), brown mustard (*Brassica juncea*) and basil (*Ocimum basilicum*) (Yin et al., 2012). The dietary intake of 0.25% carnosine plus 0.25% AA in mice is equal to 14 g carnosine plus 14 g AA for a 70-kg adult per day. Based on the natural properties, these agents at these dosages might be safe and feasible for human. Further study is necessary to verify the effects and safety of this combination at these dosages. On the other hand, the consumption of foods rich in carnosine and AA might also provide the nutritional benefits as we observed. It might be worthy to encourage diabetic subjects to consume foods rich in carnosine and AA. Besides the observed more remarkable anti-diabetic activities, this

Table 4
Plasma level or activity of glucose (mmol/l), insulin (nmol/l), triglyceride (mmol/l), total cholesterol (TC, mmol/l), ALT (IU/l), AST (IU/l), BUN (mg/dl) and creatinine (Cr, mg/dl) in normal mice (N) and diabetic mice without treatment (D), or with 0.5% Car, 0.5% AA or 0.25% Car + 0.25% AA for 6 weeks. Carnosine was added in the drink water. AA at 0.5 or 0.25 g was mixed with 99.5 or 99.75 g mouse basal diet. Values are mean ± SD, n = 8. Values with different alphabet in a column are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

	N	D	0.5 Car	0.5 AA	0.25Car + 0.25AA
Glucose, mmol/l	10.3 ± 1.0^a	27.7 ± 2.9^d	21.6 ± 1.4^c	20.7 ± 0.7^c	17.1 ± 0.9^b
Insulin, nmol/l	13.1 ± 0.9^c	5.1 ± 0.5^a	7.8 ± 0.8^b	8.7 ± 0.6^b	9.4 ± 1.0^b
Triglyceride, mmol/l	1.82 ± 0.07^a	2.88 ± 0.14^d	2.38 ± 0.09^c	2.49 ± 0.1^c	2.15 ± 0.06^b
TC, mmol/l	1.18 ± 0.09^a	1.61 ± 0.11^b	1.53 ± 0.08^b	1.55 ± 0.06^b	1.51 ± 0.12^b
ALT, IU/l	19 ± 2^a	18 ± 3^a	22 ± 4^a	23 ± 2^a	21 ± 3^a
AST, IU/l	21 ± 3^a	24 ± 4^a	20 ± 2^a	22 ± 3^a	24 ± 3^a
BUN, mg/dl	10 ± 2^a	13 ± 3^a	14 ± 2^a	10 ± 3^a	12 ± 1^a
Cr, mg/dl	0.8 ± 0.2^a	1.0 ± 0.3^a	0.9 ± 0.1^a	0.8 ± 0.3^a	1.1 ± 0.2^a

Table 5
Renal IL-1beta, IL-6, TNF-alpha level (pg/mg protein) and ROS level (RFU/mg protein) in normal mice (N) and diabetic mice without treatment (D), or with 0.5% Car, 0.5% AA or 0.25% Car + 0.25% AA for 6 weeks. Carnosine was added in the drink water. AA at 0.5 or 0.25 g was mixed with 99.5 or 99.75 g mouse basal diet. Values are mean ± SD, n = 8. Values with different alphabet in a column are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

	N	D	0.5 Car	0.5 AA	0.25Car + 0.25AA
IL-1beta, pg/mg protein	18 ± 3^{a}	257 ± 16^{d}	174 ± 9^{c}	126 ± 12^{b}	113 ± 8^{b}
IL-6, pg/mg protein	15 ± 2^{a}	221 ± 19^{e}	131 ± 10^{c}	169 ± 7^{d}	97 ± 12^{b}
TNF-alpha, pg/mg protein	23 ± 4^{a}	303 ± 22^{e}	241 ± 7^{d}	206 ± 15^{c}	173 ± 14^{b}
ROS, RFU/mg protein	0.22 ± 0.07^{a}	1.63 ± 0.16^{e}	1.02 ± 0.1^{c}	1.29 ± 0.09^{d}	0.81 ± 0.05^{b}

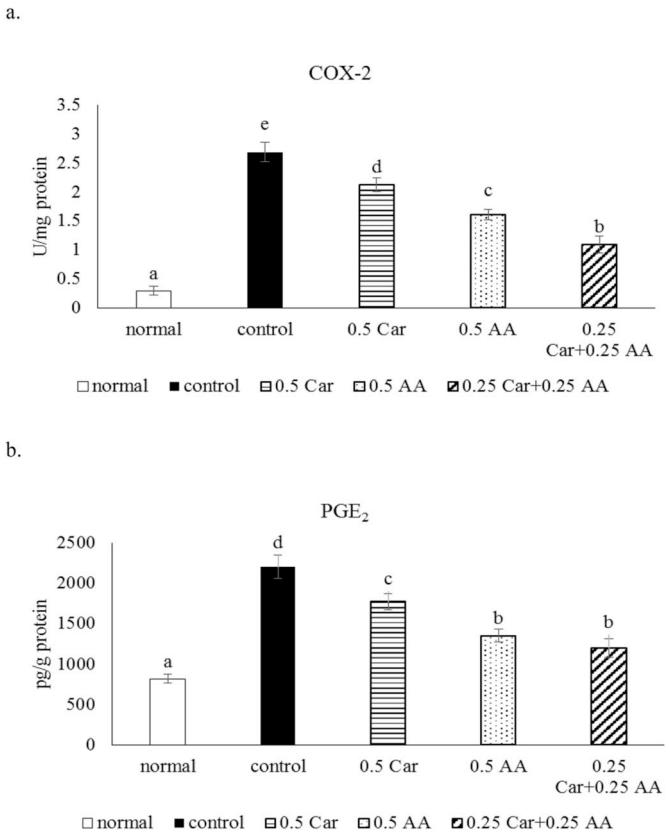


Fig. 3. Renal COX-2 activity (a, U/mg protein) and PGE_2 level (b, pg/g protein) in normal mice (N) and diabetic mice without treatment (D), or with 0.5% Car, 0.5% AA or 0.25% Car + 0.25% AA for 6 weeks. Carnosine was added in the drink water. AA at 0.5 or 0.25 g was mixed with 99.5 or 99.75 g mouse basal diet. Values are mean ± SD, n = 8. Values with different alphabet among bars are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

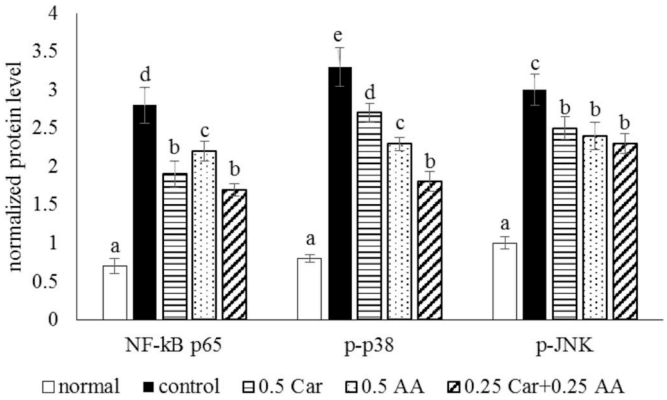


Fig. 4. Expression of NF-κB p65, phosphorylated p38 (p-p38) and phosphorylated JNK (p-JNK) in kidney of normal mice (N) and diabetic mice without treatment (D), or with 0.5% Car, 0.5% AA or 0.25% Car + 0.25% AA for 6 weeks. Carnosine was added in the drink water. AA at 0.5 or 0.25 g was mixed with 99.5 or 99.75 g mouse basal diet. Values are mean ± SD, n = 8. Values with different alphabet among bars are significantly different at $P < 0.05$ as analyzed by Dunnett's *t*-test.

combination might have other advantages due to lower AA dosage. The possible advantages include reducing economic cost because AA is relatively expensive than carnosine; and minimizing the risk of side effects from AA because AA may increase metabolic burden due to its lipophilic property. Recently, the combined effects of two phytochemicals such as quercetin plus resveratrol (Yang and Kang, 2018); or phytochemical plus clinical drug such as epigallocatechin gallate plus acarbose (Gao et al., 2013) for diabetic amelioration have attracted attention. Those previous studies and our present study suggest that the combinations from natural compounds are potentially alternative options for diabetic treatment.

In conclusion, the combination of carnosine plus asiatic acid displayed more significant protective effects for HUVE cells and diabetic mice than carnosine or asiatic acid treatment alone. The data from HUVE cells and diabetic mice indicated that this combination enhanced Bcl-2 mRNA expression, decreased DNA fragmentation, improved glycemic control, lowered TNF-alpha and ROS production, and suppressed renal COX-2 activity and p-p38 expression. Of course, further *in vivo* or even human studies are necessary to verify the effects and safety of this combination. These findings support that this combination is a promising approach for anti-diabetic treatment.

Conflicts of interest statement

All authors stated that no Conflict of Interest.

Acknowledgement

This study was partially supported by a grant from Asia University, Taichung City, Taiwan (ASIA-106-CMUH-29).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.02.027.

References

Abe, H., Okuma, E., Sekine, H., Maeda, A., Yoshiue, S., 1993. Human urinary excretion of L-histidine-related compounds after ingestion of several meats and fish muscle. Int. J. Biochem. 25, 1245–1249.
Adhikary, L., Chow, F., Nikolic-Paterson, D.J., Stambe, C., Dowling, J., Atkins, R.C., Tesch, G.H., 2004. Abnormal p38 mitogen-activated protein kinase signalling in human and experimental diabetic nephropathy. Diabetologia 47, 1210–1222.
Bitto, A., Squadrito, F., Irrera, N., Pizzino, G., Pallio, G., Mecchio, A., Galfo, F., Altavilla, D., 2014. Flavocoxid, a nutraceutical approach to blunt inflammatory conditions. Mediat. Inflamm. 790851 2014.
Brownlee, M., 2001. Biochemistry and molecular cell biology of diabetic complications. Nature (Lond.) 414, 813–820.
Ciudin, A., Hernandez, C., Simo, R., 2012. Non-invasive methods of glucose measurement: current status and future perspectives. Curr. Diabetes Rev. 8, 48–54.
Dong, S.H., Liu, Y.W., Wei, F., Tan, H.Z., Han, Z.D., 2017. Asiatic acid ameliorates pulmonary fibrosis induced by bleomycin (BLM) via suppressing pro-fibrotic and inflammatory signaling pathways. Biomed. Pharmacother. 89, 1297–1309.

Elsherbiny, N.M., Zaitone, S.A., Mohammad, H.M.F., El-Sherbiny, M., 2018. Renoprotective effect of nifuroxazide in diabetes-induced nephropathy: impact on NFκB, oxidative stress, and apoptosis. Toxicol. Mech. Methods 28, 467–473.
Fouad, A.A., Qutub, H.O., Al Rashed, A.S., Al-Melhim, W.N., 2017. Therapeutic effect of carnosine in rat model of experimental liver carcinogenesis. Environ. Toxicol. Pharmacol. 56, 10–14.
Gao, J., Xu, P., Wang, Y., Wang, Y., Hochstetter, D., 2013. Combined effects of green tea extracts, green tea polyphenols or epigallocatechin gallate with acarbose on inhibition against α-amylase and α-glucosidase in vitro. Molecules 18, 11614–11623.
Hong, Z., Hong, Z., Wu, D., Nie, H., 2016. Specific MAPK inhibitors prevent hyperglycemia-induced renal diseases in type 1 diabetic mouse model. Mol. Cell. Biochem. 419, 1–9.
Jackson, M.C., Lenney, J.F., 1996. The distribution of carnosine and related dipeptides in rat and human tissues. Inflamm. Res. 45, 132–135.
Kamei, J., Ohsawa, M., Miyata, S., Tanaka, S., 2008. Preventive effect of L-carnosine on changes in the thermal nociceptive threshold in streptozotocin-induced diabetic mice. Eur. J. Pharmacol. 600, 83–86.
Kaundal, R.K., Shah, K.K., Sharma, S.S., 2006. Neuroprotective effects of NU1025, a PARP inhibitor in cerebral ischemia are mediated through reduction in NAD depletion and DNA fragmentation. Life Sci. 79, 2293–2302.
Kumral, A., Giriş, M., Soluk-Tekkeşin, M., Olgaç, V., Doğru-Abbasoğlu, S., Türkoğlu, Ü., Uysal, M., 2016. Beneficial effects of carnosine and carnosine plus vitamin E treatments on doxorubicin-induced oxidative stress and cardiac, hepatic, and renal toxicity in rats. Hum. Exp. Toxicol. 35, 635–643.
Lee, A.S., Kim, J.S., Lee, Y.J., Kang, D.G., Lee, H.S., 2012. Anti-TNF-α activity of Portulaca oleracea in vascular endothelial cells. Int. J. Mol. Sci. 13, 5628–5644.
Li, J., Chen, Y.J., Quilley, J., 2005. Effect of tempol on renal cyclooxygenase expression and activity in experimental diabetes in the rat. J. Pharmacol. Exp. Therapeut. 314, 818–824.
Liao, H.H., Zhu, J.X., Feng, H., Ni, J., Zhang, N., Chen, S., Liu, H.J., Yang, Z., Deng, W., Tang, Q.Z., 2017. Myricetin possesses potential protective effects on diabetic cardiomyopathy through inhibiting IκBα/NFκB and enhancing Nrf2/HO-1. Oxid. Med. Cell. Longev. 2017, 8370593.
Momtaz, S., Hussein, A.A., Ostad, S.N., Abdollahi, M., Lall, N., 2013. Growth inhibition and induction of apoptosis in human cancerous HeLa cells by Maytenus procumbens. Food Chem. Toxicol. 51, 38–45.
Ozdoğan, K., Taşkın, E., Dursun, N., 2011. Protective effect of carnosine on adriamycin-induced oxidative heart damage in rats. Anadolu Kardiyol. Derg. 11, 3–10.
Peiretti, P.G., Medana, C., Visentin, S., Giancotti, V., Zunino, V., Meineri, G., 2011. Determination of carnosine, anserine, homocarnosine, pentosidine and thiobarbituric acid reactive substances contents in meat from different animal species. Food Chem. 126, 1939–1947.
Purnell, J.Q., Hokanson, J.E., Marcovina, S.M., Steffes, M.W., Cleary, P.A., Brunzell, J.D., 1998. Effect of excessive weight gain with intensive therapy of type 1 diabetes on lipid levels and blood pressure: results from the DCCT. Diabetes control and complications trial. J. Am. Med. Assoc. 280, 140–146.
Puttarak, P., Panichayupakaranant, P., 2012. Factors affecting the content of pentacyclic triterpenes in Centella asiatica raw materials. Pharm. Biol. 50, 1508–1512.
Ramachandran, V., Saravanan, R., Senthilraja, P., 2014. Antidiabetic and antihyperlipidemic activity of asiatic acid in diabetic rats, role of HMG CoA: in vivo and in silico approaches. Phytomedicine 21, 225–232.
Schelman, W.R., Andres, R.D., Sipe, K.J., Kang, E., Weyhenmeyer, J.A., 2004. Glutamate mediates cell death and increases the Bax to Bcl-2 ratio in a differentiated neuronal cell line. Brain Res. Mol. Brain Res. 128, 160–169.
Schilling, G., Wood, J.D., Duan, K., Slunt, H.H., Gonzales, V., Yamada, M., Cooper, J.K., Margolis, R.L., Jenkins, N.A., Copeland, N.G., Takahashi, H., Tsuji, S., Price, D.L., Borchelt, D.R., Ross, C.A., 1999. Nuclear accumulation of truncated atrophin-1 fragments in a transgenic mouse model of DRPLA. Neuron 24, 275–286.
Si, L., Xu, J., Yi, C., Xu, X., Wang, F., Gu, W., Zhang, Y., Wang, X., 2014. Asiatic acid attenuates cardiac hypertrophy by blocking transforming growth factor-β1-mediated hypertrophic signaling in vitro and in vivo. Int. J. Mol. Med. 34, 499–506.
Wu, W.T., Mong, M.C., Yang, Y.C., Wang, Z.H., Yin, M.C., 2018. Aqueous and ethanol extracts of daylily flower (Hemerocallis fulva L.) protect HUVE cells against high glucose. J. Food Sci. 83, 1463–1469.
Yang, D.K., Kang, H.S., 2018. Anti-diabetic effect of cotreatment with quercetin and resveratrol in streptozotocin-induced diabetic rats. Biomol. Ther. (Seoul). 26, 130–138.
Yin, M.C., Lin, M.C., Mong, M.C., Lin, C.Y., 2012. Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. J. Agric. Food Chem. 60, 7697–7701.