Research Article

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Cardiac protection of functional chicken-liver hydrolysates on the high-fat diet induced cardio-renal damages via sustaining autophagy homeostasis

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Abstract

BACKGROUND: Cardio-renal syndrome (CRS) is an integrative problem related to chronic malnutrition, obesity, etc. Amino acids and peptides are regarded as protective and essential for tissues. Pepsin-digested chicken liver hydrolysates (CLHs), which are made from the byproducts of the poultry industry, are amino-acid based and of animal origin, and may be protective against the myocardial and renal damage induced by a high-fat diet (HFD).

RESULTS: Our results showed that CLHs contain large quantities of anserine, taurine, and branched-chain amino acids (BCAAs), and supplementing the diet with CLHs reduced ($P < 0.05$) weight gain, liver weight, peri-renal fat mass / adipocyte-area sizes, serum total cholesterol (TC), aspartate aminotransferase (AST), and low-density lipoprotein cholesterol (LDLC) levels in HFD-fed mice but increased ($P < 0.05$) serum high-density lipoprotein cholesterol (HDLC) levels. By histological analyses, CLHs alleviated ($P < 0.05$) renal lipid deposition and fibrosis, as well as cardiac fibrosis and inflammation of HFD-fed mice. Meanwhile, increased ($P < 0.05$) inflammatory and fibrotic cytokines levels in the myocardia of the HFD-fed mice were downregulated ($P < 0.05$) by CLH supplementation. Regarding autophagy-related protein levels, protective effects of CLHs on the myocardia against HFD feeding may result from the early blockade of the autophagy pathway to prevent autophagosome accumulation.

CONCLUSION: Functional CLHs could be a novel food ingredient as a cardio-renal protective agent against a high-fat dietary habit in a niche market.
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Keywords: autophagy; cardio-renal damage; chicken liver hydrolysate; high-fat diet; myocardium

INTRODUCTION

In 2017, cardiovascular disease (CVD) was the leading cause of death globally, accounting for 31.80% of all deaths, which is almost double the second leading cause of death (cancers, 17.08%).[1] A high-fat diet (HFD)-fed rodent model has been widely used to imitate long-term exposure to the risk factors associated with metabolic syndrome. The disturbance of lipid homeostasis leads to a cardiac lesion, which is often associated with human renal dysfunction.[2,3] Recently, the term cardio-renal syndrome (CRS) has been used to indicate different clinical conditions of the heart together with kidney dysfunction. Metabolic CRS induced by an HFD is known as secondary CRS (also known as type 5 CRS), which is affected by systemic problems such as diabetes mellitus and insulin resistance.[4,5]

In recent decades, the pathological process of metabolic-associated CVDs has been demonstrated to be related to autophagy.[2] It has been indicated that HFD-induced signal cascades influence an autophagy signal.[6] The autophagy-lysosomal pathway is essential for cellular metabolic homeostasis. There are two situations involved in autophagy-lysosomal pathway: (i) the redundant cellular components, including proteins, nucleic acids, carbohydrates, and lipids, are degraded into precursors and molecules for survival under nutrient deprivation, and (ii) the excess nutrients, damaged proteins or organelles could be removed via the autophagic degradation pathway.The autophagy-lysosomal pathway is an intrinsic degradation process where the cellular components, excess nutrients, damaged proteins as well as organelles are broken down into precursors and molecules for survival. Participating proteins could be classified into three

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functional groups: (i) detectors of external factors and initiators of the autophagy process, e.g., ATG1 and mTOR; (ii) structural proteins for primary membrane and carrier proteins for degrading materials, e.g. P62, ATG8, and LC3B-II; and (iii) markers for matured autolysosome, e.g., Rab7. It was revealed that an HFD stimulates autophagy initiation but retards autophagosome maturation, which might result in autophagosome accumulation due to the suppression of RAB7 expression.[7] Hence, a rescue of HFD-induced RAB7 suppression could lead to the normal cardiac regulation in mice.[7]

Although the broiler liver is regarded as cheap and useless in the processing stream, it contains many nutrients, such as proteins and the trace minerals selenium (Se) and manganese (Mn).[8] Many reports have revealed bioactive hydrolysates with origins in food-proteins.[9,10] Our previous studies have also reported that pepsin-digested chicken liver hydrolysates (CLHs) contain plenty of anserine (a dipeptide containing β-alanine and 1-methylhistidine), taurine, branched-chain amino acids (BCAAs) (i.e., isoleucine, leucine, and valine), and some anti-oxidant amino acids (i.e., aspartic acid, glutamic acid, and histidine), which could enhance anti-oxidative activity against chronic alcohol consumption and have an anti-fibrotic effect.[8,11,12] The chicken is the best source among all meat sources of imidazole rings containing dipeptides (carnosine and anserine).[13,14] Several reports mentioned that anserine demonstrates strong anti-oxidative and anti-glycation effects.[13] Taurine supplementation can also attenuate liver injury, hepatic steatosis, and hyperlipidemia; moreover, it also improves the redox homeostasis and muscle function.[15] Branched-chain amino acids have also been proven to affect energy metabolism in the heart by regulating glucose and the fatty acid metabolism.[16] For example, leucine could regulate mammalian cardiac mTOR signaling, which directly dominates protein synthesis, insulin sensitivity, and autophagy.[17] Increasing dietary leucine intake could chronically reduce weight gain and hypercholesterolemia in HFD-fed mice.[18] Our previous report indicated that functional CLHs obtained from pepsin digestion could offer a lipid-lowering effect against an HFD.[19] However, further investigation is required of the effect of our functional CLH on an autophagy-signaling pathway of myocardia against chronic HFD consumption. This study was therefore designed to offer scientific evidence that CLH supplementation attenuates cardio-renal injury in HFD-fed mice.

MATERIALS AND METHODS

Materials

The chicken livers (Ross broiler) were purchased from Charming Food International Marketing Co., Ltd (Taichung, Taiwan), which met the Certified Agriculture Standards in Taiwan. The harvested livers were kept and transported at a temperature below −20°C. First, antibiotic residues were examined at the Experiment Station Chemical Laboratories of National Animal Industry Foundation (Pingtung, Taiwan), and the livers were all found to be antibiotic free (data not shown). Pepsin (400 U mg^{-1}, Sigma-Aldrich, Inc., St Louis, MO, USA) was used to hydrolyze the liver. All other chemicals used in this study were of analytical grade. The functional CLHs were manufactured as in our previous study, and the manufacturing process has been patented.[8,20]

Free amino-acid profiles and carnosine/anserine contents of the CLHs

The free amino-acid profile and carnosine / anserine content of CLHs was analyzed at the Analysis Research and Service Center of the Food Industry Research and Development Institute (Hsin-Chu City, Taiwan). This assay was performed with the Amino Acid Analyzer (Hitachi L8800 amino acid analyzer, Hitachi High-Technologies Co., Tokyo, Japan). Briefly, the lyophilized CLH was homogenized with 20 mL trichloroacetic acid (7%, v/v) for 2 min, and then filtered (No.1 filter paper, 55 mm, Advantec, Tokyo Roshi Kaisha Ltd, Japan). The precipitates were homogenized twice with trichloroacetic acid as described above. Finally, the data were reported as milligrams of amino acid or carnosine / anserine per 100 g of lyophilized CLHs.

Animal and diets

Animal use and protocol were reviewed and approved by the National Taiwan University Care Committee (IACUC No.: 106-EL-00093). Forty male, 8-week-old C57BL/6 mice were purchased from the Laboratory Animal Center of the National Taiwan University. The environmental parameters of the animal house were as follows: temperature, 22 ± 2 °C and light / dark cycle, 12/12 h. All mice were randomly assigned to one of the following groups (n = 8 per group): (i) Control, control diet; (ii) HFD, high fat diet (fat provides 46.5% of calories); (iii) HFD + CLH1X, high-fat diet and CLHs (approximately 170 mg kg^{-1} BW); (iv) HFD + CLH3X, high-fat diet and CLHs (approximately 510 mg kg^{-1} BW); (v) HFD + CNT, high-fat diet and L-carnitine (CNT) (approximately 500 mg kg^{-1} BW). Two mice with an ear-taq (No. 1 or No. 2) were housed in one cage. The dosage of CNT was determined as described in a previous report.[21] The diet formula was based on AIN-93 M (supplementary Table 1), and the diet for each treatment was modified by adjusting either its fat, CLHs, or CNT content. Chicken liver hydrolysates or CNT was mixed with basal feed ingredients (casein was partially replaced to keep a constant total protein amount). For consistent consumption of CLHs or CNT in each treatment, their concentration in weekly feeds must be normalized based on the group average feed intake and the body weights in the previous week. It could avoid the bios from intensive weight changes and ingestion differences due to different energy contains. Water and diets were provided *ad libitum* for 1 week before the experiment (basal diet only) and during the experimental period (assigned diets). The body weight per mouse, and feed and water intake per cage, was recorded weekly, and the average daily feed and water intake was calculated per mouse.

$$\begin{aligned}&\textit{Concentration of additive CLHs or}\\&\textit{CNT in modified feeds}\,(\%)\,\textit{in each week}\\&=\frac{\textit{body weight}\,(kg)_{\textit{previous week}}\times\textit{reference dose}\,(g/kg\,BW)}{\textit{average feed intake}\,(g)_{\textit{last week.}}}\end{aligned}$$

Collection of serum, liver, abdominal fat, and feces of experimental mice

During the trial period, blood was collected from each mouse at the first, 10th, and 20th weeks of the experiment. Serum total cholesterol (TC) and triglyceride (TG) levels were monitored. At the end of the experiment, all mice were weighed and fasted for 8 h, and then sacrificed by CO_2 asphyxiation. Blood samples were collected by orbital sinus and kept at room temperature for 1 h. Sera were obtained after centrifugation (Model# 3700, Kubota Co., Tokyo, Japan) at 3000×*g* and 4 °C for 10 min. The heart, liver, kidney, and abdominal adipose tissues of each mouse were removed and weighed individually. The left kidney, half heart (left of long-axis plane dissection which contains the left ventricle), and half peri-renal adipose tissue were placed into a 10% formaldehyde solution for histological analysis,

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and other remnants were immediately immersed in liquid N_2 and then stored at −80 °C for subsequent analysis.

Serum biochemical values

Serum total protein (TP), globulin (GLO), blood urea nitrogen (BUN), creatinine (Cre), uric acid (UA), Na^+, K^+, aspartate aminotransferase (AST), alanine aminotransferase (ALT), TC, TG, HDLC, and LDLC levels were assayed using an auto biochemistry analyzer (TBA 120FR TOSHIBA ChemistryAnalyzer, Toshiba Technology, Tokyo, Japan) and their corresponding kits. The atherosclerosis index (AI) of each mouse was calculated as the ratio of LDLC divided by HDLC.

Serum lipid peroxidation level and anti-oxidant capacity

Thiobarbituric acid reactive substances (TBARS), Trolox equivalent anti-oxidant capacity (TEAC), and reduced glutathione (GSH) levels in sera were assayed using previously described methods.[12] The TBARS values were used as a lipid peroxidation marker. The TEAC assay was based on the measurement of $ABTS^+$ scavenging ability, and indicates the total anti-oxidative capability. Finally, the reduced GSH level was detected from the level of the functional thiol group in the GSH.

Histological analyses of adipose tissue, kidney, and heart

All tissue blocks, slides and stains were prepared according to the methods described in our previous study.[12] The adipocyte size in peri-renal adipose tissues was quantified using ImageJ-Adiposoft (National Institutes of Health, Bethesda, MD, USA). Microphotographs (100×) of them were analyzed according to the official guideline of ImageJ-Adiposoft. The program could count the total adipocyte numbers and show the area (μm^2) of each cell under the field of vision, and the average of those parameters in each group was calculated and displayed. The quantification of redness in Sirus red-stained slides of the myocardium (40×), renal cortex (100×), and outer medulla (100×) was analyzed using ImageJ (National Institutes of Health). The thresholds of each tissue microphotograph were established according to their background, morphology, and color density; the minimum and maximum thresholds were therefore given as 145/168 (myocardium), 120/159 (cortex), and 90/154 (medulla), respectively. Redness indicates injury and fibrotic characteristics for each tissue sample.

Western blotting and enzyme-linked immunosorbent assay (ELISA)

The procedures for sample preparation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transfer, and antibody hybridization were conducted as described in our previous study.[12] Electrophoresis was performed using Bio-Rad's Mini-Protean Tetra Cell System (BioRad Laboratories Inc., Hercules, CA, USA), and the proteins were transferred via either Bio-Rad's Trans-Blot Turbo Semi-Dry Transfer System or the Criterion Wet Transfer Blotter. The protein signals on the membrane were complemented using an enhanced ECL kit (#RPN2235 or #RPN2232, GE) with the Bio-Rad ChemiDoc imaging system. Image Lab software (BioRad Laboratories Inc., Hercules, CA, USA) was used to quantify the optical density of protein bands relative to the β-tubulin band. The antibodies used in this study were: COX2 (#12282, Cell Signaling Technology Inc., Danvers, MA, USA), CD36 (#EPR6573, Abcam, Cambridge, UK), αSMA (#ab5684, Abcam), MMP9 (#AB19016, Millipore Co., Billerica, MA, US), MMP2 (#4022, Cell Signaling Technology), P62 (#EPR4844, Abcam), LC3 (#2775, Cell Signaling Technology), Rab7 (#9367, Cell Signaling Technology), and β-tubulin (#2128, Cell Signaling Technology). All antibodies were diluted 1:1000 with Tris buffered saline with Tween 20 (TBST buffer). The myocardial tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and transforming growth factor (TGF)-β levels were measured using an enzyme-linked immunosorbent assay (ELISA) and conducted according to the commercial manufacturer's instructions (TNF-α, BioLegend, Inc., San Diego, CA, USA; IL-6, IL-1β, and TGF-β kits, R&D system, Minneapolis, MN, USA).

Statistical analysis

The experiment was conducted using a completely randomized design. When a significant difference ($P < 0.05$) among groups was detected using a one-way ANOVA, differences between treatments were further distinguished by using the least significant difference (LSD) test. All statistical analyses of data were conducted using SAS (SAS Institute Inc., Cary, NC, USA, 2002).

Table 1. The free amino acid composition in CLHs

Amino acids	Amount (mg per 100 g lyophilized CLHs)
L-Arginine	619.37
L-Histidine	189.84
L-Isoleucine	352.12
L-Leucine	830.31
L-Lysine	789.49
L-Methionine	272.05
L-Phenylalanine	400.62
L-Threonine	457.44
Tryptophan	77.59
L-Valine	612.83
Total EAA	4601.66
Total BCAA	1795.26
L-Alanine	772.46
β-Alanine	33.03
γ-Aminobutyric acid	1.86
DL-3-Aminoisobutyric acid	35.46
Asparagine	18.63
L-Aspartic acid	677.01
L-Cystathionine	18.01
Ethanolamine	20.27
L-Glutamic acid	1132.65
Glycine	443.57
DL-plus allo-δ-hydroxylysine	5.33
L-Hydroxyproline	23.61
L-Ornithine	28.2
o-Phosphoserine	75.15
L-Proline	605.16
L-Serine	578.72
Taurine	308.10
L-Tyrosine	305.87
Total NEAA	5083.09
Carnosine	N.D.
Anserine	126.02
Dipeptide	126.02

N.D., not detectible; EAA, essential amino acid; NEAA, non-essential amino acid; BCAA, branched-chain amino acid.

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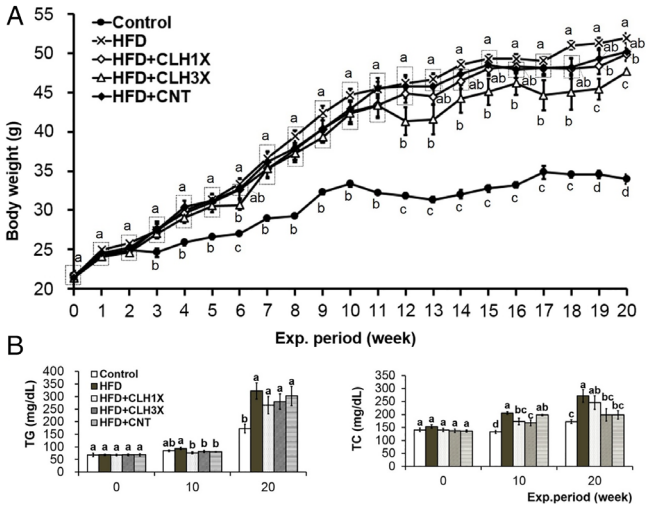

Figure 1. The body weights and serum lipids of experimental mice. (a) The body weights, (b) serum TG and TC of mice in the experimental periods. The data are given as mean ± SEM (n = 8). Mean values or data bars in each experimental period without a common letter are significantly different ($P < 0.05$).

RESULTS

Effects of CLHs on growth performance, serum biochemical analyses, and organ weight in HFD induced mice

According to the free amino-acid profile of CLHs in this study, the content of free anserine, taurine, isoleucine, leucine, and valine was 126.02, 308.10, 352.12, 830.31, and 612.83 mg per 100 g lyophilized CLH, respectively while the total free essential amino acid (EAA) and BCAA content were 4601.66 and 1795.26 mg per 100 g lyophilized CLHs, respectively (Table 1). In addition, the content of *β*-alanine, the precursor of carnosine, was 33.03 mg per 100 g lyophilized CLH. Regarding the growth performance of the mice, the average final body weights in HFD, HFD + CLH1X, HFD + CLH3X, and HFD + CNT (51.97 ± 0.58, 49.91 ± 0.76, 47.70 ± 0.27, and 50.19 ± 1.12 g) were significantly higher ($P < 0.05$) than those of the control group (33.99 ± 0.55 g), whereas CLH-supplemented groups were significantly lighter ($P < 0.05$) than the HFD group (Fig. 1(a)). The body weight of the HFD + CLH3X group was consistently lower ($p < 0.05$) than that of the HFD group from the 12th week of the experimental period. The daily feed intakes of the HFD, HFD + CLH1X, HFD + CLH3X, and HFD + CNT groups (3.17 ± 0.08, 3.17 ± 0.12, 3.20 ± 0.13, and 3.28 ± 0.18 g per mouse per day) decreased slightly compared to the control group (3.89 ± 0.13 g per mouse per day) while the water intakes were not ($P > 0.05$) different among the groups (data not shown). At the end of the trial period, hyperlipidemia was successfully induced in HFD-fed mice, but lower ($p < 0.05$) serum TC levels were observed in the HFD + CLH3X and HFD + CNT groups, and serum TG levels of CLH or CNT supplemented groups tended to be lower than that of the HFD group (Fig. 1(b)). Regarding organ weights, kidney mass remained consistent among all groups (Table 2). The HFD feeding increased ($P < 0.05$) heart and liver weight, but CLH or CNT supplementation attenuated ($P < 0.05$) those increases. In the blood biochemical analyses (Table 2), the HFD group had a higher ($P < 0.05$) AST level than the control group, and the AST levels of CLH or CNT supplemented groups were alleviated ($P < 0.05$) and were even similar to that of the control group. A similar pattern was observed for the ALT levels, but there were no ($P > 0.05$) differences among the groups. Focusing on the LDLC and HDLC levels, CLH supplementation increased ($P < 0.05$) the HDLC values of

Table 2. The organ weights, blood biochemical values, and serum anti-oxidative capacities of experimental mice

	Control	HFD	HFD + CLH1X	HFD + CLH3X	HFD + CNT
Organ weights					
Heart (g)	0.15 ± 0.00b	0.19 ± 0.01a	0.16 ± 0.01b	0.16 ± 0.01b	0.15 ± 0.01b
Liver (g)	1.14 ± 0.03c	2.23 ± 0.16a	1.76 ± 0.16b	1.39 ± 0.18b	1.81 ± 0.06b
Kidney (g)	0.37 ± 0.02a	0.36 ± 0.01a	0.39 ± 0.01a	0.32 ± 0.05a	0.34 ± 0.01a
Blood biochemical analysis and atherosclerosis index					
TP (g dL^{-1} serum)	5.56 ± 0.07a	5.46 ± 0.12a	5.46 ± 0.09a	5.46 ± 0.13a	5.66 ± 0.06a
GLO (g dL^{-1} serum)	2.23 ± 0.03a	2.30 ± 0.04a	2.29 ± 0.05a	2.43 ± 0.07a	2.35 ± 0.04a
BUN (mg dL^{-1} serum)	20.25 ± 0.39a	21.64 ± 0.15a	19.98 ± 0.30a	20.51 ± 0.26a	20.21 ± 0.39a
Cre (mg dL^{-1} serum)	0.33 ± 0.02a	0.34 ± 0.02a	0.33 ± 0.02a	0.35 ± 0.02a	0.36 ± 0.02a
UA (mg dL^{-1} serum)	1.99 ± 0.06a	1.91 ± 0.05a	1.81 ± 0.08a	2.01 ± 0.14a	1.88 ± 0.11a
Na^+ (mmol L^{-1} serum)	145.50 ± 0.19a	146.14 ± 0.24a	145.50 ± 0.33a	146.00 ± 0.27a	146.25 ± 0.25a
K^+ (mmol L^{-1} serum)	4.69 ± 0.02a	4.66 ± 0.02a	4.68 ± 0.03a	4.68 ± 0.04a	4.63 ± 0.03a
AST (U L^{-1} serum)	107.13 ± 9.72c	175.50 ± 14.33a	136.88 ± 4.98bc	142.63 ± 10.06b	120.38 ± 14.09bc
ALT (U L^{-1} serum)	47.50 ± 3.62a	63.75 ± 3.95a	49.13 ± 2.14a	56.38 ± 4.95a	59.00 ± 7.72a
LDLC (mg dL^{-1} serum)	50.00 ± 1.34b	62.50 ± 3.34a	56.75 ± 5.41ab	63.88 ± 5.15a	47.63 ± 1.27b
HDLC (mg dL^{-1} serum)	70.88 ± 3.26b	69.38 ± 3.20b	99.00 ± 6.36a	96.00 ± 6.10a	78.88 ± 3.47b
LDLC/HDLC	0.72 ± 0.04b	0.91 ± 0.04a	0.57 ± 0.04c	0.66 ± 0.02bc	0.61 ± 0.04c
Serum anti-oxidative capacities					
TBARS (nmole MDA eq. mL^{-1} serum)	17.19 ± 0.70c	51.48 ± 3.82a	23.89 ± 1.61b	23.36 ± 1.40bc	26.09 ± 2.72b
Reduced GSH (nmole mL^{-1} serum)	55.74 ± 1.73ab	52.75 ± 1.45b	60.25 ± 2.32a	60.45 ± 1.20a	55.35 ± 2.06ab
TEAC (nmole mL^{-1} serum)	0.99 ± 0.02b	0.94 ± 0.01c	1.00 ± 0.02ab	1.00 ± 0.01b	1.04 ± 0.01a

The data are given as means ± SEM (n = 8). Mean values in each parameter without a common letter are significantly different ($P < 0.05$).

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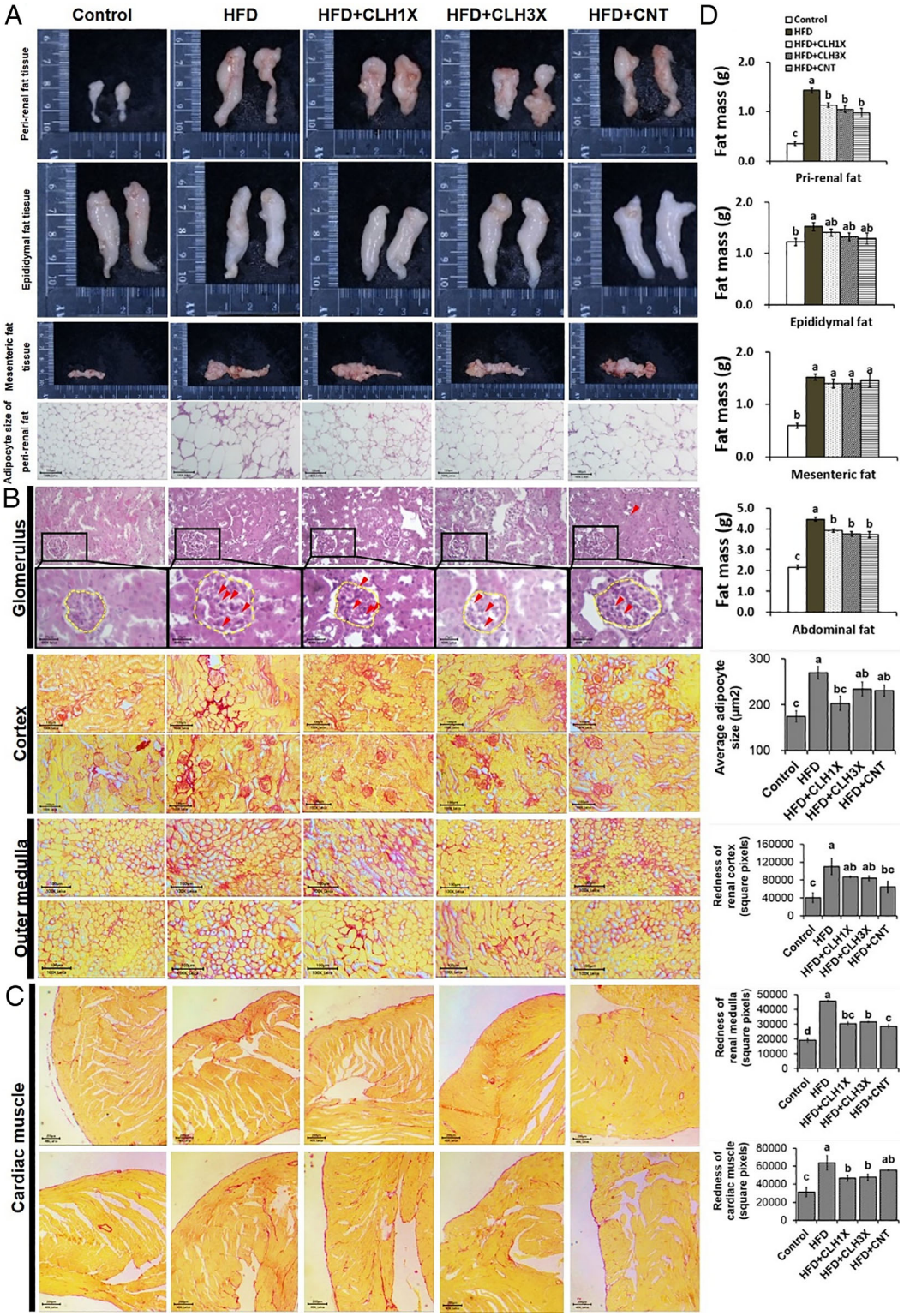

Figure 2. Appearance of fat tissue and pathohistological analyses of experimental mice. (a) Appearance of abdominal fat, including peri-renal, epididymal, and mesenteric fat tissues. According to the results of the fat mass in each fat subtype (peri-renal, epididymal, and mesenteric fat tissues), the morphologies of peri-renal fat tissues were then demonstrated via H&E stainings. The photos are shown with 100× magnification. (b) Glomerulus is demonstrated with 100× and 400× magnification, and the yellow dash-line shows the area of the glomerulus, where red arrows indicate the lipid droplet dislocation. Sirius red staining of the renal cortex and outer medulla are shown with 100× magnification. (c) The Sirius red stainings of the myocardium are shown with 40× magnification (the scale bar indicates 200 μm). (d) The weights of abdominal fat tissues, including peri-renal, epididymal, and mesenteric fat tissues, are shown. The quantification of adipocyte-area size as well as redness quantification of the Sirius red stained tissue slides were performed using ImageJ. The data are presented as means ± SEM (n = 8). Data bars in each tested parameter without a common letter are significantly different ($P < 0.05$).

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HFD-fed mice, whereas CNT supplementation decreased ($P < 0.05$) the LDLC value of HFD-fed mice. Furthermore, an increased ($P < 0.05$) AI (LDLC/HDLC) was calculated in the HFD group compared to that in the control group while those of the CLH- or CNT-treated groups were significantly decreased ($P < 0.05$) (Table 2). Other blood biochemical values were not ($P > 0.05$) different among the groups. Finally, in the HFD-fed mice, the serum TBARS was increased, GSH was slightly decreased, and TEAC values were depleted ($P < 0.05$) compared to those of the control group (Table 2). Then, the CLH or CNT supplementation decreased ($p < 0.05$) the serum TBARS values of HFD fed mice but enhanced ($p < 0.05$) reduced GSH levels and TEAC values in HFD-fed mice.

Effects of CLHs on obesity and renal morphology in HFD induced mice

The mass of abdominal fat tissues, including peri-renal fat, epididymal fat, and mesenteric fat, are shown in Fig. 2(a) and (d), and each of the subtypes was increased ($P < 0.05$) by HFD feeding. The attenuated effects ($P < 0.05$) were only observed in the peri-renal fat tissues in the CLH or CNT supplemented groups (Fig. 2(a) and (d)). Moreover, CLH or CNT supplementation reduced ($P < 0.05$) the average adipocyte sizes of peri-renal fat tissues. Lipid accumulation (red arrows) was observed in the renal glomerulus of HFD-fed groups, but less lipid accumulation resulted from CLH and CNT supplementation (Fig. 2(b)). Furthermore, the illustrations of Sirius red staining indicated the renal fibrosis (cortex and outer medulla parts) in the HFD-fed mice obviously, but CLH or CNT supplementation ameliorated the renal fibrosis (Fig. 2(b)). According to the quantification analysis of the redness, the CLH or CNT supplementation significantly decreased ($P < 0.05$) the redness of renal outer medulla, but there was only a tendency toward reduced redness of the renal cortex in the CLH supplemented groups (Fig. 2(d)).

Effects of CLHs on myocardial damage in HFD induced mice

Histological results illustrated that redder areas (collagen and fibrotic tissues) could be observed in the myocardia of HFD-fed mice (Fig. 2(c)), which is consistent with the quantification result (redness levels) of cardiac muscle (Fig. 2(d)). Supplementation with CLH lessened red areas and decreased ($P < 0.05$) the redness levels of cardiac muscle (Fig. 2(c)&D). Furthermore, the increased ($P < 0.05$) levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6

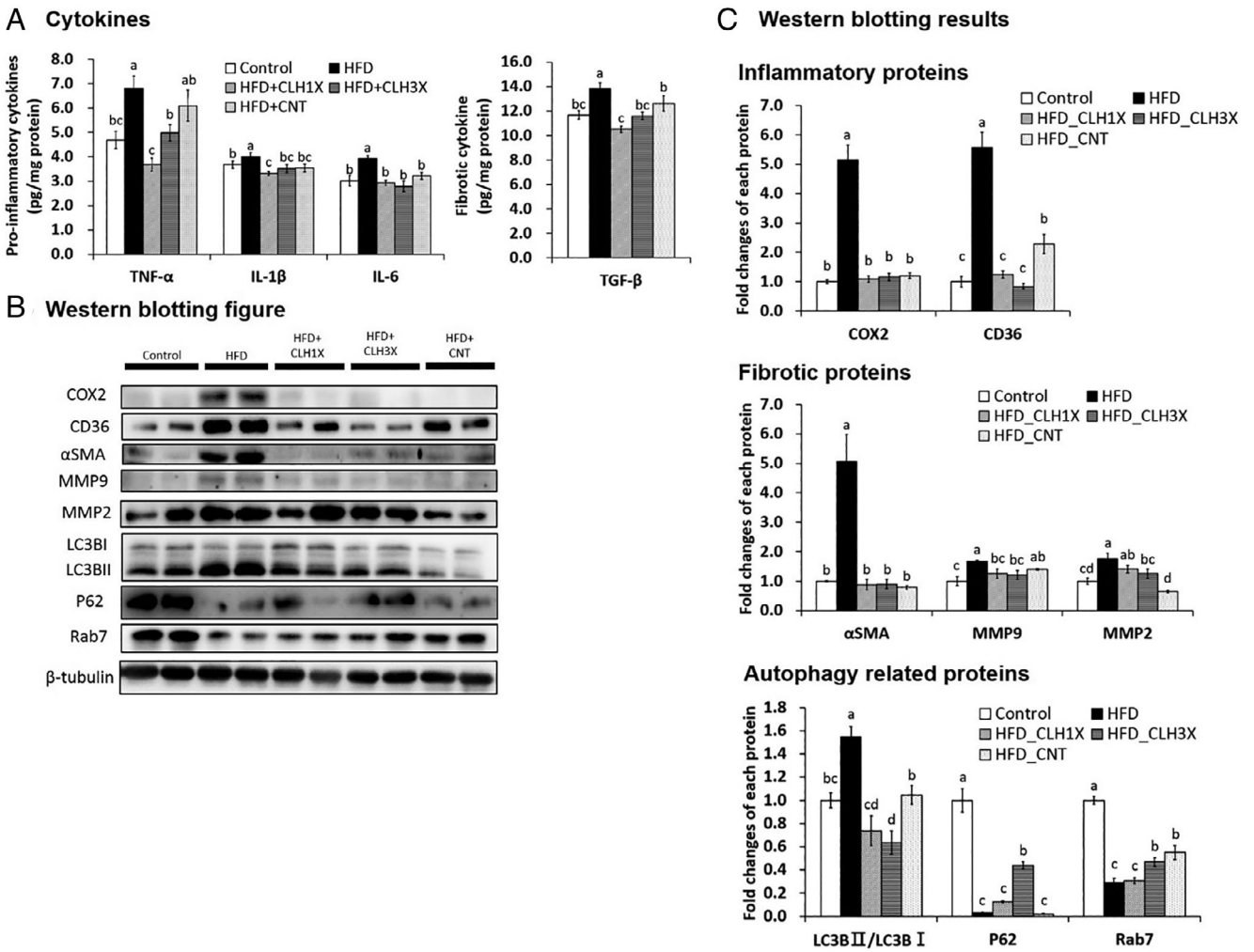

Figure 3. The effects of CLHs on target protein expressions in the myocardia of experimental mice. (a) The cytokine (pro-inflammatory and fibrotic) concentrations of myocardia. (b) Western blotting illustration. (c) The quantification results of western blotting, including pro-inflammatory, fibrotic, and autophagy-related proteins. The data are given as mean ± SEM (n = 8). Data bars in each tested parameter without a common letter are significantly different ($P < 0.05$).

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levels) and fibrotic cytokine (TGF-β) in the myocardia of the HFD group compared to those of the control group were downregulated ($P < 0.05$) by CLH or CNT supplementation (TNF-α: HFD + CNT *vs*. HFD, $P > 0.05$) (Fig. 3(a)). The pattern of inflammation, fibrotic, and autophagy-related proteins is illustrated in Fig. 3(b). In comparison with the control group, enhanced ($P < 0.05$) inflammatory and fibrotic related protein levels (i.e., COX2, CD36, αSMA, MMP9, and MMP2) were also detected in the HFD group, but they are downregulated ($P < 0.05$) by CLH or CNT supplementation. Regarding the autophagy-related protein levels, compared to those of HFD group, the P62 level was only higher ($P < 0.05$) in the HFD + CLH3X group, and a decreased ($P < 0.05$) ratio of LC3βII/LC3βI was detected in the CLH or CNT supplemented groups (Fig. 3(c)). The Rab7 protein levels were downregulated ($P < 0.05$) in the HFD-fed groups compared to the control group, but the HFD_CLH3X and HFD_CNT groups showed a higher ($P < 0.05$) protein level than the HFD group (Fig. 3(c)).

DISCUSSION

The profile indicated that CLHs, including anserine, EAA, taurine, L-anserine, L-ornithine, and β-alanine possessed a high nutrition value (Table 1). The chicken meat was regarded as a good source for peptides containing an imidazole ring (carnosine and anserine).[13,14] The report indicated that the anserine content of chicken meat was about 116.5 to 336.7 mg per 100-g dried weight, which depends on the portion, and the anserine content in this study, 126.02 mg per 100-g lyophilized CLHs, was consistent with this.[13] Through the hydrolysis and lyophilization of liver tissues, the concentration of anserine was obviously maintained in our CLHs, and similar as that in chicken meat (Table 1). As mentioned above, BCAA affects the heart regulation through its signaling transduction on myocardial metabolism; thus, its deficiency promoted heart failure.[22] Leucine supplementation retarded the systemic lipid and glucose homeostasis dysregulation, thereby countering the adverse effects of an HFD.[23] Furthermore, cardiac leucine dominated the metabolism of BCAAs in the heart through mTOR complex-1 signaling.[24] The BCAA content in CLHs was almost 40% (w/w) among EAAs, and L-leucine was up to 18% (Table 1). It has been reported that taurine decreases the risk of coronary heart disease through several mechanisms, and there is evidence that taurine could alleviate dyslipidemia by enhancing hepatic lipid homeostasis.[25] In CLHs, taurine was also measured (308 mg free-form type taurine per 100 g CLHs). The high L-arginine and ornithine levels in CLHs also indicated that they could promote nitric oxide metabolism, and their metabolites could stimulate protein synthesis in muscle and attenuate physical fatigue.[26,27] Moreover, the β-alanine supplement could result in cardiac protection and ameliorate metabolic syndrome.[28] Hence, it is reasonably hypothesized that CLHs are characterized as a cardiac protection due to its anserine content and free amino-acid profile. The raised serum TC and TG levels were a crucial cause of CVDs. L-Carnitine (CNT), an amino acid derivative, has been used for clinical supplements in both pre- and post-surgery cases, and it has also been proven to be protective against HFD-induced metabolic disease.[21,29,30] The similar changed pattern of serum TC and TBARS levels were observed in HFD-fed mice supplemented with CLHs. The increased serum TC levels in HFD-fed mice were attenuated by supplementing CLHs (Fig. 1), which might result in the decreases of serum TBARS values (Table 2). Supplementation with CLH or CNT affected serum LDLC and HDLC levels, and the CLH decreased AI by raising HDLC, although CNT tended to reduce the LDLC (Table 2). It was speculated that the hypolipidemic effect of CLHs was possibly due to regulating effects of taurine on hepatic lipogenesis, fatty-acid β-oxidation, energy expenditure, and cholesterol metabolism.[11] According to the clinical report, the increased heart weight indicated myocardium hypertrophy. The data indicated that CLH or CNT supplementation decreases the elevated heart mass of HFD group compared to that of the control group (Table 2). However, it was indicated that the HFD feeding can cause metabolic syndrome but insufficient to induce cardiac dysfunction in mice.[31] The primary protective mechanism of protein hydrolysates may occur due to its anti-oxidative properties, and a similar observation was also demonstrated in many animal food-protein sources *in vitro* or *in vivo*.[32] Likewise, CLH supplementation reduced the serum TBARS values; Likewise, CLH supplementation reduced the serum TBARS values; meanwhile, reversed the reduced GSH levels and TEAC values in sera of HFD-fed mice (Table 2). These findings indicated that CLHs alleviated systemic injury and oxidative stress. This anti-oxidative effect of CLHs might result from amino acid content (L-glutamic acid, L-aspartic acid, L-leucine, L-valine, L-isoleucine, and L-histidine) or imidazole rings containing dipeptides (anserine) in CLHs (Table 1); moreover, anti-oxidative minerals (e.g., Mn, Zn, and Se) were also detected in CLHs in our previous study.[8] As mentioned, the cardiac protective effect of CLHs may be attributed to a combination of its hepatic lipid-lowering effect and systemic anti-oxidation.

Nowadays, it was proved that the gut microbiome is different between obese and non-obese individuals. The intestinal dysbiosis would alter the lipid metabolism of the host, which leads to obesity.[33] The intestinal microbial structure and composition could shift rapidly due to a diet such as the HFD, and the transit time was on average 2–3 days only in humans.[34] Furthermore, it was demonstrated that the interaction among nutrients, bacteria, and immune system is importantly regulated in the intestine.[35] Intestinal immune and barrier functions could be modulated by functional amino acid such as BCAA, tryptophan; thus, the nutrient manipulated the gut microbiome not only from energy source but also gut immune system.[35,36] In addition, anti-inflammatory or antibacterial ingredients and peptides derived from the Lactobacillus app. could improve the diet-induced obesity by enhancing intestinal health, such as epithelial barrier integrity.[37,38] Hence, the gut microbiome may be influenced by CLH supplementation, which may alter the inflammation as well as metabolism in experimental mice. The investigation based on this assumption deserves a further study. An accumulated clinical evidence pointed out that the obesity exacerbates a cardiac injury. For example,an excessive adipose accumulation could increase the circulating blood volume and cardiac output, thus leading hypertension, hypertrophy, and heart failure in the most clinical cases.[39] Furthermore, peri-renal fat tissue was strongly associated with adverse metabolic risk, including CVD.[40] Although HFD was insufficient to induce cardiac dysfunction and heart failure in mice, the results showed evidence of myocardial-specific fibrosis and lipid deposition as significant pathological outcomes in this study. Our data suggested that weight gain (Fig. 1(a)), hyperlipidemia (Fig. 1(b)), and abdominal fat mass (Fig. 2(a) and (d)) in the HFD group indicated obesity, but anti-obesity effects ($P < 0.05$) were demonstrated in CLH or CNT supplemented groups. Weight loss had been proven to reduce the mortality rate and incidence of coronary heart disease, and improved hypertension in the clinical cases.[39] The anti-obesity effect of our CLHs could also be attributed to the cardiac

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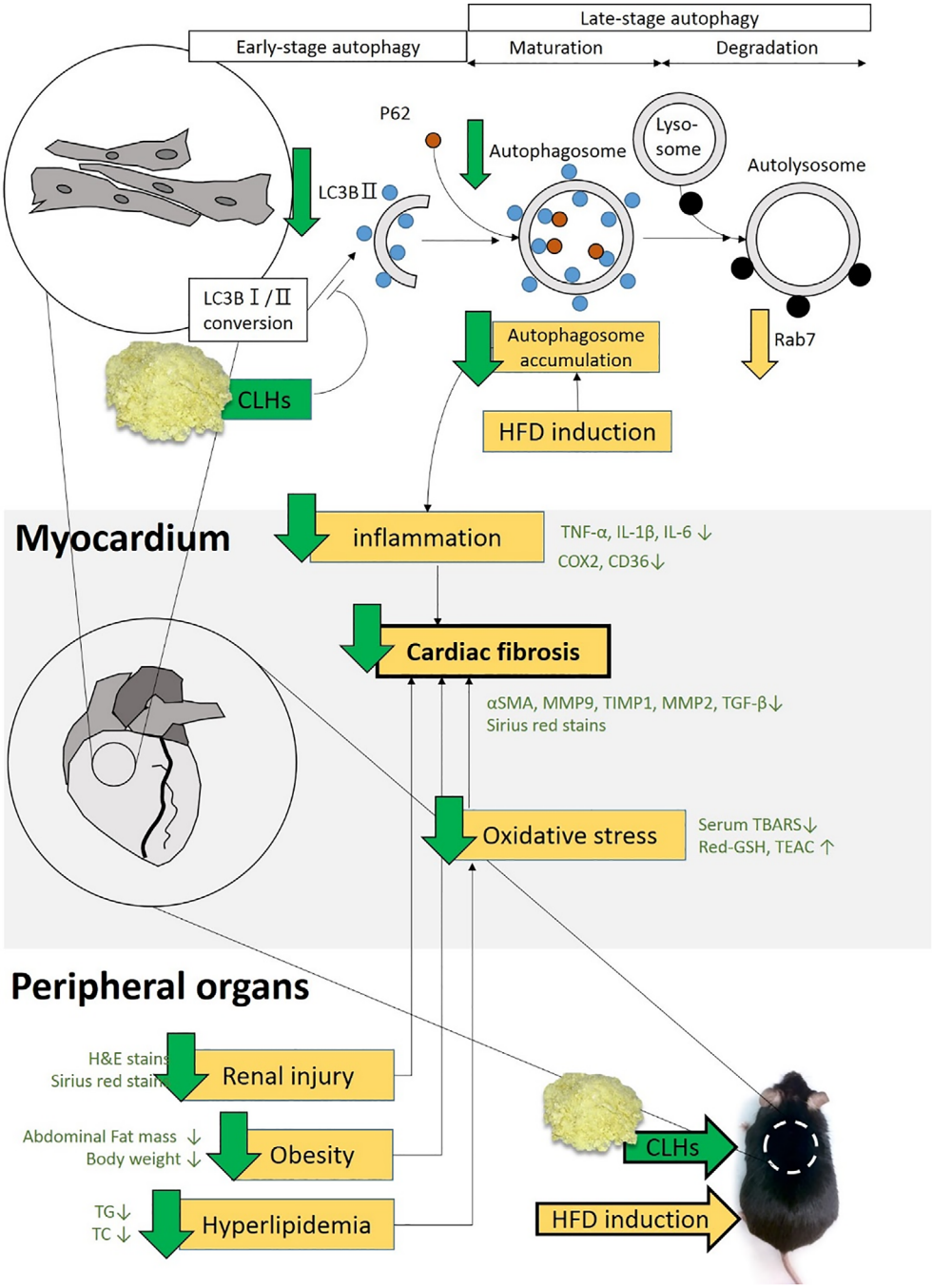

Figure 4. The protective effects of CLHs in the myocardial and peripheral organs against HFD induction. The HFD-induced metabolic disorders are indicated with yellow text boxes, whereas the attenuated effects of CLH supplementation are shown with green arrows and texts. The three parts of the schematic diagram demonstrate the impact of peripheral organs and myocardia.

protective effect. However, the body weights of HFD-fed hamsters with or without CLHs remained no change during 8 weeks of experiment due to the insufficient experimental period, but the lipid-lowing effects were shown obviously *via* regulating the lipid metabolism and energy expenditure pathway according to our former investigation.[11] In the other hand, the trend of body weights (Fig. 1) at the last week was incompatible to those of other protective effects such as anti-oxidative capability (Table 2) and histological analysis (Fig. 2) in this study. Altogether, it was reasonable to assume that protective effects of CLHs are synergistic under the CLH supplementation.

Cardiovascular disease was also strongly associated with renal function because the kidney might directly influence the total blood volume and blood pressure. It was reported that a long-term high-fat / sucrose diet promoted lipid deposition, glomerular hypertrophy, and apoptosis in the kidney.[3,41] In this study, a similar HFD induced pathological outcomes, including lipid deposition and hypertrophy in the renal glomerulus areas, as well as fibrosis in the renal cortex and outer medulla (Fig. 2(b) and (d)). Interestingly, the development and progression of hyperlipidemia-induced glomerular injury were due to oxidative stress, and the protective effects of CLH supplementation might be associated with its

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systemic antioxidative property.[8,42] The intravenous administration of a large number of amino acids protected the renal tubule system where both reabsorption and anion balance are retained within chronic renal disease patients.[43] Moreover, L-histidine, L-arginine, and L-ornithine supplements were protective against oxidative stress damage and inflammation in chronic kidney disease patients, and those amino acids were abundant in CLHs (Table 1).[44–46]

Myocardial fibrosis was a significant contributor and linked obesity, left ventricular dysfunction, and heart failure.[47] Moreover, tissue remodeling and wound healing played a crucial role in injury and fibrosis progression. The matrix metallopeptidases (MMPs) were the primary mediators of extracellular matrix remodeling, and both MMP2 and MMP9 were strongly associated with severe morphological changes and dysfunctions in cardiomyopathies.[48] As a result, CLH supplementation not only decreased the levels of inflammatory and fibrotic proteins in myocardia of HFD-fed mice (Fig. 3(a) and (b)) but also attenuated MMP2 and MMP9 protein expression (Fig. 3(c)). Our previous study reported that the anti-fibrotic effect of CLHs is obtained in TAA-induced liver injured mice.[12] Previous studies indicated that taurine owns hepatoprotective, anti-oxidative, and lipid-lowering modulation while taurine is detectable in CLHs.[15,16] Its protective effect was correlated with the lower serum LDLC/HDLC and TBARS values, and higher reduced GSH and TEAC levels probably (Table 2), which may be attributed to the anti-oxidative amino acids (e.g. L-glutamic acid, L- aspartic acid, BCAAs, L-histidine), or L-anserine in CLHs. Based on the results in the former study, anserine can improve the neurovascular-unit dysfunction and anti-inflammation.[49] The anserine in CLHs (126.02 mg per 100-g lyophilized CLHs) may contribute to the protective effect.[49] Recently, a growing number of studies reported that autophagy plays an essential role in the process of myocardial fibrosis. High-fat-diet-induced cardiac hypertrophy and lipotoxicity was associated with an impaired autophagic response, where the accumulation of autophagosome and an excess of lipid led to endoplasmic reticulum (ER) stress as well as apoptosis in HFD-fed mice.[50,51] Myocardial autophagosome maturation is disrupted in HFD induced obesity, insulin resistance, and dyslipidemia; it might be mediated through downregulation of Rab7.[7] However, BCAA, especially L-leucine, modulated autophagy, and the metabolic pathway via mTOR signal cascades, and the amount of BCAAs was also assayed (1795.26 mg per 100-g lyophilized CLHs) in our CLHs.[18] Hence, L-leucine and BCAA may play an upstream modulator in protective effects of CLHs. The protein levels of Rab7 were significantly decreased in the myocardia of HFD-fed groups; moreover,the conversion from LC3BI to LC3BII seemed to be upregulated in the HFD group. Those phenomena may indicate autophagosome accumulation (Fig. 3(C)). It was demonstrated that an accumulation of initial autophagy protein, P62 (Fig. 3(c)), which was known as a marker of an early-stage blockade.[5] Although CLH or CNT supplementation could not rescue the HFD-induced Rab7 repression (Fig. 3(c)), it interrupted the initiation of the autophagy pathway by downregulating ($P < 0.05$) the activation of the structural protein LC3B (Fig. 3C). To sum up, CLH supplementation attenuated HFD-induced autophagosome accumulation by early stage blockade of the autophagy pathway in the myocardium, thereby helping to maintain autophagy homeostasis. Resveratrol also protects muscle cells against palmitate toxicity by ameliorating autophagic flux, which sheds light on the relationship between cardiac protection and autophagic homeostasis.[52] However, the exact role and impact of sustained cardiac autophagic homeostasis in HFD-induced metabolic diseases still needs further investigation.

CONCLUSION

In this study, CLH supplementation could attenuate a cardiac pathological progression in a chronic HFD intake (Fig. 4). The hypolipidemic effect, anti-obesity, and renal protective effects of our CLHs were shown in peripheral organs by the results of histological and blood biochemical analysis. Global oxidative stress was also attenuated. The anti-inflammatory and anti-fibrotic effects of our CLHs were also proven by western blotting in the myocardia, and those effects may be related to the early blockade of the autophagy pathway to prevent the HFD-induced autophagosome accumulation. The evidence showed that the protective outcomes of our CLHs are due to systemic and synergistic effects. Nevertheless, the connection between the functional CLHs and myocardial protection may result from bioactive ingredients in CLHs, e.g., anserine, taurine, glutamic acid, aspartic acid, BCAAs, β-alanine, and histidine. The metabolic impact of CLHs and the exact role of sustained autophagy homeostasis in all organs and tissues warrants further investigation in future studies to elucidate its potential in nutraceutical or medical applications.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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