BJPS

Brazilian Journal of Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20510

Article

Decreased body-fat accumulation and increased vasorelaxation to glyceryl trinitrate in middle-aged male rats following six-weeks consumption of coconut milk protein

Jomkarn Naphatthalung[1], Pilaipan Chairuk[1], Somruedee Yorsin[5], Kanyanatt Kanokwiroon[2,4], Nisaudah Radenahmad[3], Chaweewan Jansakul[1*]

[1]Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat-Yai, Thailand, [2]Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat-Yai, Thailand, [3]Department of Anatomy, Faculty of Science, Prince of Songkla University, Hat-Yai, Thailand, [4]The Excellent Research Laboratory of Cancer Molecular Biology, Prince of Songkla University, Hat-Yai, Thailand, [5]Faculty of Medicine, Princess of Naradhiwas University, Narathiwas, Thailand

We investigated whether coconut milk protein (CMP) contributes to the beneficial effects of coconut milk consumption on cardiovascular health markers previously found in middle-aged rats. CMP was isolated and precipitated from dried fresh coconut milk, then gavaged (1 g/kg) to middle-aged male rats for six weeks; control rats received distilled water. Compared to controls, CMP caused decreased body fat and lipid accumulation in liver cells and the platelet count. CMP did not affect basal blood pressure or heart rate in anesthetized rats. Vascular responsiveness to phenylephrine, DL-propargylglycine (PAG), acetylcholine or sodium nitroprusside was unaffected, but vasorelaxation to glyceryl trinitrate (GTN) increased. Effects of ODQ on vasorelaxation to GTN were similar in both groups. Expression of blood vessel eNOS, CSE and sGC was normal. The cyclic guanosine monophosphate (cGMP) level of CMP-treated rats was normal but addition of GTN increased cGMP and NO concentration more in CMP-treated rats than in controls, an effect unaltered by addition of diadzin. Taken together, CMP appears partially responsible for the improvement in cardiovascular health markers caused by coconut milk in middle-aged male rats.

Keywords: Coconut protein. Blood vessel. Liver lipid. NO. Glyceryl trinitrate.

INTRODUCTION

Globally, cardiovascular disease is the leading cause of death. Its etiology is multifactorial but age and nutrition are two of the most important risk factors. As population aging is accelerating in nearly all countries of the world (NIA, 2019; UN, 2020). It is important that everyone should establish good lifestyle options, and eat a diet of healthy foods with suitable macro- and micronutrients to prolong their healthy life and to age without increasing the healthcare burden.

Advancing age is associated with increased intra-abdominal visceral fat accumulation (Huffman, Barzlai, 2009; Kotani *et al*., 1994; Ponti *et al*., 2020; Tchkonia *et al*., 2010; Zamboni *et al*., 2014). This leads to endothelial dysfunction (Arcaro *et al*., 1999; Chait, den Hartigh, 2020; Pazos, 2020) due to decreased vascular eNOS expression, and thereby diminishes nitric oxide (NO) production (Novella *et al*., 2013) which represents the early stage of pathophysiological changes in the development of cardiovascular disease (Bhayadia *et al*., 2016; Marchesi *et al*., 2000; Rudolph *et al*., 2007).

Coconut, *Cocos nucifera*, is one of the most economically important palm species and is cultivated

***Correspondence:** C. Jansakul. Faculty of Traditional Thai Medicine. Prince of Songkla University. Hat-Yai, Thailand 90110. Phone: 66-074-286824. E-mail address: chaweewan.j@psu.ac.th. ORCID: https://orcid.org/0000-0002-5703-3677

mainly for its nutritional endosperm from which coconut milk and coconut oil are its main products (Gwee, 1988). Coconut milk is a common culinary ingredient of Southeast Asian, Indian, Sri Lankan cuisines, as well as in Brazilian, Caribbean and Polynesian food (D'Amato, Fasoli, Righetti, 2012). The main constituents of coconut milk are lipids (coconut oil, 41.5 %) with a small amount of proteins (4.5 %) and carbohydrates (5.2 %) (Pehowich, Gomes, Barnes, 2000), or about 80 %, 19 % and 5 %, respectively in dried fresh coconut milk (Jansakul *et al*., 2018).

To date, there has been only limited scientific investigation of the cardiovascular effects of the consumption of coconut kernel. Padmakumaran Nair, Rajamohan, Kurup (1999) were the first to demonstrate that the consumption of fresh coconut kernel alone or together with coconut oil by young rats caused lower serum total cholesterol (TC) and a lower lipid profile but higher serum triglyceride. Later, Ekanayaka *et al.* (2013) studied the effect in human subjects and found that eight-week consumption of coconut-milk porridge caused a lower plasma level of LDL with increased HDL cholesterol. Recently, Vijayakumar *et al.* (2018) reported that when normal healthy human-beings consumed a diet enriched with 100 g fresh coconut for 90 days they experienced a reduction in plasma glucose and body weight. Meanwhile our research group studied middle-aged male rats and found that the consumption of pure dried fresh coconut milk caused up-regulation of blood vessel endothelial nitric oxide synthase (eNOS) and cystathionine γ-lyase (CSE) protein expression, which resulted in an attenuation of the contractile response to phenylephrine and potentiate relaxation to acetylcholine on the rats' thoracic aortic rings, with a decreased fasting plasma glucose level (Jansakul *et al*., 2018). Further, these effects were very similar to those resulting from the consumption of dried fresh coconut milk oil except that the retroperitoneal fat accumulation was increased (Naphatthalung *et al*., 2019), which indicates that there must be other active components in the coconut milk that counteract fat accumulation from the coconut milk oil.

Coconut protein is the second major component of coconut milk which has been found to have beneficial effects on cardiovascular risk parameters, such as decreased levels of serum cholesterol, triglycerides and phospholipids, and decreased lipogenesis in the liver and intestines of rats (Padmakumaran Nair, Rajamohan, Kurup, 1998), as well as reduced hyperlipidemia and peroxidative effect in rats fed a high fat/cholesterol diet (Salil, Rajamohan, 2001). In addition, Mini, Rajamohan (2002) found a cardio-protective effect in rat isoproterenol-induced myocardial infarction. Salil, Nevin, Rajamohan (2011) discovered anti-diabetic activity, which might occur via the arginine-NO pathway in rats with alloxan- and streptozotocin-induced diabetes, and this led to pancreatic beta cell regeneration. Thus, it is possible that coconut milk protein might be responsible for the restoration of animal body weight caused by dried fresh coconut milk consumption in middle-age male rats found by Jansakul *et al*. (2018). So far, there have been no reports of the effect of coconut protein consumption on blood pressure and vascular functions. Therefore, the present study aimed to investigate whether coconut milk protein is responsible for the visceral-fat reduction effect, as well as effects on other cardiovascular risk parameters seen in research involving the consumption of dried fresh coconut milk. In this experiment, dried fresh coconut milk was prepared as previously described, and the coconut milk protein (CMP) fractions were isolated in order to explore the effects of the consumption of CMP in middle-aged male rats.

MATERIAL AND METHODS

Coconut milk protein preparation

Fresh mature coconut kernel was grated and compressed using an electric screw press to obtain a large sample of fresh aqueous coconut milk. The milk was then filtered through a cloth filter followed by lyophilization to obtain fresh dried coconut milk, which was kept at -20 ˚C until used.

The dried fresh coconut milk was centrifuged (2500 g) at room temperature to achieve pure coconut milk oil (CMO), comprising 70 % of the dried coconut milk, and precipitate. The precipitate was dissolved in distilled water and the protein was precipitated with

50 % ethanol. The protein precipitate was collected and the ethanol removed in an evaporator before lyophilization to obtain dried crude CMP, yielding 19 % of the dried coconut milk. In the remainder of the text the abbreviation CMP is used to represent crude coconut milk protein, except at some points in the text where emphasis is required, the word "crude" is used before the abbreviation CMP.

The crude CMP was characterized for its protein, oil and sugar content. Its trace oil content was determined using the hexane defatting method, by liquefying 100 mg CMP with 100 µL distilled water and then stirring it thoroughly with 1 mL hexane. The hexane solution was separated and the hexane removed by evaporation to obtain the coconut oil. The total sugar content was determined by the classical colorimetric method (DuBois *et al*., 1956) and the protein content was determined by Bradford assay. Each experiment was repeated four times with different samples (n = 4).

The CMP was analyzed for its protein composition by 1D and 2D-gel electrophoresis and its total amino acid composition was determined by an in-house method (HPLC-precolum-AccQ). Tag, using the service of the Central Instrument Facility, Faculty of Science, Mahidol University, Bangkok, Thailand).

Pharmacological studies

Middle-aged (13-14 month old) male Wistar rats were bought from the National Laboratory Animal Center, Mahidol University. The animals were housed in controlled environmental conditions at 25 ˚C on a 12 h dark and 12 h light cycle and allowed access to standard food (Perfect Companion Group Co. Ltd, Thailand), and tap water *ad libitum*. The animal methods employed in this study were approved by the Prince of Songkla University Animal Ethics Committee (Ethic Number: Ref. 06/57). The investigation conformed to the Guide for the Care and Use of Laboratory Animals (CIOMS Guidelines). The rats were randomly divided into two groups, with six animals in each type of experiment except for the Western blot analysis in which 4 rats were used for each group. The experimental group was treated by oral administration of 1 g/kg of rat body weight (hereafter g/kg) coconut protein with the control group animals receiving distilled water (DW) once a day for six weeks. The body weight and 24 h food intake (one day before receiving oral gavage of CMP or DW) were recorded at day 0, and again every consecutive 7th day over the 6-week period. Thus, at the end of treatment the age of the rats was about 15-16 months.

Effects of the CMP or DW treatment on the basal blood pressure and on the haematology and clinical biochemical analysis

The same methods as previously described by Yorsin *et al*. (2014) were used. At the end of the 6-week CMP or DW treatment, after fasting for 13-15 h, the rats were anaesthetized with thiopental sodium (60 mg/kg). Their blood pressure and heart rate were recorded via the right common carotid artery by a polyethylene catheter connected to a polygraph, the data being collected after a 40-min equilibration period.

Following this, the rat was sacrificed by decapitation with a guillotine and two tubes of blood samples were collected. The first was used for the analysis of the glucose and lipid levels by enzymatic methods using an automatic chemistry analyser routinely operated at the Prince of Songkla University Hospital. The other was sent to the haematology laboratory for the analysis of its total blood count measured by an automated haematology analyser.

Effects of CMP or DW treatment on internal organs and lipid accumulation

The decapitated rats were dissected as previously described (Yorsin *et al*., 2014). The heart, lung, liver, adrenal gland, kidney and testes, and the visceral fats from the epididymis, testis, retroperitoneal, and subcutaneous fats were removed and weighed.

Two pieces of liver (middle lobe) were cut, embedded into a cryostat gel, and the sections (20 µm thick) were stained with oil red O (0.5 % in absolute propylene glycol), and mounted with glycerine jelly for observation by light microscopy. The aortic arch was collected and was cleared of adhering fat and connective tissue before

being stained with oil red-O, using the same method as for the liver tissue. The oil red O of each slide of the liver tissue and of the aortic arch was extracted with 1 mL of 100 % dimethyl sulfoxide (DMSO), and its absorbance was measured at 520 nm. The concentration of the oil red O was obtained from the standard curve of known concentrations of oil red O in 100 % DMSO (μg/mL). The area of a thin whole-liver section and of the aortic arch was measured using the Auto CAD 2005 program. The amount of the accumulated liver lipid was expressed in terms of μg/mL/cm^2 of the thin liver-tissue section area and the aortic arch.

Preparation of the thoracic aortic rings

The thoracic aorta was removed from the decapitated rat and placed in oxygenated Krebs-Henseleit solution at 37 ˚C and the adhering connective tissue was removed. Six adjacent rings of 4-5 mm in length were cut. Each aortic ring was mounted with two stainless steel hooks in a 20 mL organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4 \cdot 7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37 ˚C and bubbled with carbogen (95 % O_2 and 5 % CO_2 gas mixture). One of the hooks was fixed at the bottom and the other was connected to a transducer in order to record the isometric tension using a polygraph. The tissues were equilibrated for 60 min under a resting tension of 1 g and the bath solution was replaced with pre-warmed oxygenated Krebs-Henseleit solution every 15 min.

At the end of the equilibration period, each aortic ring was tested for the viability of the endothelium by precontraction with phenylephrine (3 μM) until the response reached a plateau (5-8 min), and then the addition of acetylcholine (30 μM). Endothelial viability was judged by a > 65 % vasorelaxation back to the tension generated by the ring before the addition of the phenylephrine. The preparations were then washed several times with Krebs-Henseleit solution, and allowed to fully relax for 45 min and the basal tension of the thoracic aortic rings was adjusted to the optimal tension of 2 g before the experimental protocol began.

Effects of the CMP or DW treatment on the pharmacological vascular functions

Effects on contraction to phenylephrine and role of NO and H_2S

After equilibration, the contractile response to the cumulative concentration-response (*C-R*) curve of phenylephrine was obtained. This was followed by several washings, and the aortic rings were allowed to fully relax for 50 min. They were then pre-incubated with L-NA for 40 min, and the second *C-R* curve to phenylephrine was then obtained. After repeated washings and re-equilibrations for 40 min, the third *C-R* curve to phenylephrine was obtained in the presence of DL-propargylglycine (PAG) and L-NA.

Effects on relaxation to acetylcholine, glyceryl trinitrate, sodium nitroprusside and role of NO, H_2S and sGC

Another set of aortic rings was precontracted with phenylephrine (3 μM) for 10-15 min (plateau phase) following which the cumulative dilator *C-R* curves to acetylcholine were determined. After repeated washing to remove the agonists and re-equilibration for 40 min, the second *C-R* curve to acetylcholine was obtained in the presence of DL-propargylglycine (PAG).

Using the above protocol and separate sets of aortic rings, the cumulative dilator *C-R* curves to glyceryl trinitrate (GTN) or sodium nitroprusside were obtained in the presence of L-NA alone and then together with PAG. Using other sets of aortic rings, the cumulative dilator *C-R* curves to GTN were performed in the presence of L-NA alone and then together with 0.1 μM or 1 μM 1H-(1,2,4) Oxadiazolo (4,3-a) quinoxalin-1-one (ODQ), sequentially.

eNOS, CSE and sGC western blot analysis

The thoracic aorta of the CMP- treated groups and the distilled water control groups (n = 4) were obtained in order to measure the expression level of the enzymes, eNOS, CSE and sGC. After removal of the adhering connective tissue, the blood vessel was cut

into small rings and kept at -70 ˚C until used. Protein extraction from the tissues and western blot analysis were carried out as previously described (Yorsin *et al.*, 2014). Briefly, the total proteins were extracted from the homogenized tissue of each animal in lysis RIPA buffer (GE Healthcare: 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 0.5 mM EDTA containing a protease inhibitor cocktail). The protein lysate of each animal was centrifuged and the supernatant was used to quantitate the protein content by Bradford assay. Protein at 50 µg was run on 12 % SDS-polyacrylamide gel electrophoresis. Then, the protein bands were transferred onto nitrocellulose membranes. The membranes were blocked with 5 % low fat dry milk in TBS-T (Tris buffer saline- 0.1 % Tween 20) for 1 h, followed by primary antibody incubation against eNOS (1:250), CSE (1:1,000), sGC (1:500) and β-actin (1:1,000) dissolved in 1 % low fat dry milk in TBS-T overnight at 4 ˚C The rabbit eNOS and rabbit β-actin antibodies used were from Cell Signalling (USA); the mouse CSE was from Abnova (USA) and the mouse sGC was from Enzo Life Sciences Inc. (USA). The membranes were then incubated with HRP-conjugated rabbit IgG (1:5,000) for eNOS and β-actin; mouse IgG antibody (1:5,000) for CSE and sGC antibodies. The membranes were incubated with a chemiluminescence detection kit (Pierce, Rockford, USA) and the protein signal was detected by Fusion FX5XT spectra/ Superbright (Vilber Lourmat). The protein normalization was performed by measuring the band intensities of eNOS, CSE, sGC and actin using the Fusion Capt Advanced Quantitation Analysis program (Vilber Lourmat Sté, Collégien, France). The intensity value of each protein was divided by the intensity of actin from the same sample in order to obtain the protein/actin ratio. Four independent experiments were performed.

Cyclic Guanosine monophosphate (cGMP) measurement by ELISA

Thoracic aortic rings from the CMP-treated groups and the DW control groups (n = 4) were incubated in tissue baths containing L-NA (3 mM) alone (control) or with 3 µM GTN, for 10 min. The aortic rings from each incubating medium were collected and kept at -70 ˚C until used. Each frozen aortic ring was chopped on ice and homogenized in 0.1 M HCl (0.4 g of tissue in 1 mL of 0.1 M HCl). The lysates were centrifuged (14,000 rpm for 10 min) and the supernatants were collected for cyclic nucleotide ELISA (Enzo Life Science, USA), performed according to the manufacture's guidelines. The cyclic nucleotide content was normalized to obtain the total protein content of each sample by Bradford assay.

Nitric oxide concentration measurement

Thoracic aortic rings from the CMP- treated groups and the DW control groups (n = 4) were incubated in tissue-baths containing L-NA (3 mM) alone (control) or together with (1) 3 µM GTN or (2) 3 µM GTN plus diadzin (3 mM) for 10 min. The aortic rings from each incubating medium were collected and kept at -70 ˚C until used. Each frozen aortic ring was chopped on ice and homogenized in 0.1 M HCl (0.4 g of tissue in 1 mL of 0.1 M HCl). The lysates were centrifuged (14,000 rpm for 10 min) and the supernatants were collected for nitric oxide determination using Griess reagent (Sigma, USA), the nitrite content was calculated using sodium nitrite as the standard, and the total protein was determined by Bradford assay. The NO content was normalized to obtain the total protein content of each sample.

Drugs

The following drugs were used: Acetylcholine chloride, N^G-nitro-L-arginine (L-NA), norepinephrine, phenylephrine hydrochloride, DL-propagylglycine (PAG), pentobarbital, diadzin, sodium nitroprusside, sodium nitrite, Griess reagent and oil red O from Sigma, USA. GTN was obtained from Mycomed, Denmark. The acetylcholine chloride and phenylephrine were dissolved in a solution containing NaCl 9 g/L, NaH_2P0_4 0.19 g/L and ascorbic acid 0.03 g/L.

Statistical analysis

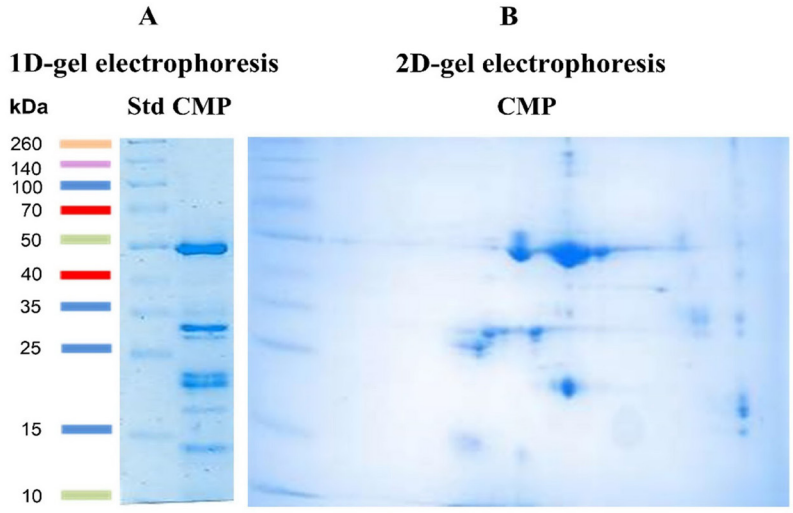
The results were expressed as the mean ± standard error of the mean (SEM) (n = 6 for vascular function study and n = 4 for western blot analysis). Statistical differences

were determined by unpaired *t*-test or by one-way analysis of variance (ANOVA), followed by Tukey's range test, using GraphPad Prism 5.00. A P value < 0.05 was considered to indicate a significant difference between values.

RESULTS

Composition of crude CMP and its amino acid composition

The crude CMP was composed of 81 ± 0.8 % protein, 9.7 ± 0.8 % oil and 6.9 ± 0.8 % sugar (n = 4). Based on 1D and 2D gel electrophoresis, the CMP contained at least 12 different proteins, and the main one had a molecular weight of about 50 kDa (Figure 1A and B). The total amino acid composition (%) of the CMP is shown in Figure 1C. CMP contains all of the essential amino acids in the range of 1-3 %, except for methionine and tryptophan for which detection is limited in this method. The other seven non-essential amino acids were also found to be in the same range as that of the essential amino acids except arginine and glutamic acid, which were found to represent about 7-9 %.



C

Total amino acid composition of CMP (mg/100 mg of protein)

Essential aa		Non-essential aa	
Isoleucine	1.3	Aspartic acid	3.9
Leucine	2.7	Serine	2.1
Lysine	2.1	Glutamic acid	9.4
Methionine	-	Glycine	1.9
Phenylalanine	1.9	Alanine	1.7
Threonine	1.4	Arginine	7.0
Tryptophan	-	Proline	1.5
Valine	2.0		
Histidine	1.0		
Tyrosine	0.9		

FIGURE 1 - Protein composition of coconut milk protein (CMP) analyzed by 1D (A) and 2D-gel electrophoresis (B) and its total amino acid (aa) composition (C) analyzed by in-house method (HPLC-precolum-AccQ. Tag).

Effects of CMP treatment on body weight, food intake, blood biochemistry, internal organ weight and body fat accumulation

In comparison to the DW control group, there was no difference in animal body weight, food intake, blood biochemistry or any of the elements of the complete blood cell count except for the platelet count which was found to be lower in the CMP-treatment group (Figure 2, Suppl. Tables I-II and Table I). None of the internal organ weights was found to be increased after treatment with CMP (Table III). CMP caused decreased accumulation of mesentery, retroperitoneal and subcutaneous fat (Table II), as well as decreased fat in the liver cells and internal wall of the aortic arch (Figures 3 and 4).

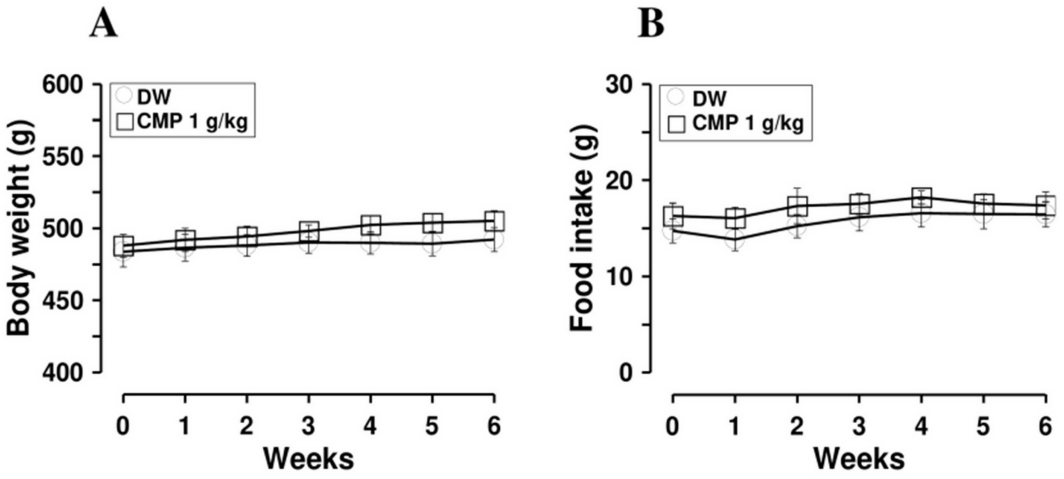


FIGURE 2 - Effects of CMP (1 g/kg) or distilled water (DW) consumption by the middle-aged male rats on body weight (left) and food intake (right). Each point represents mean ± SEM of 6 rats.

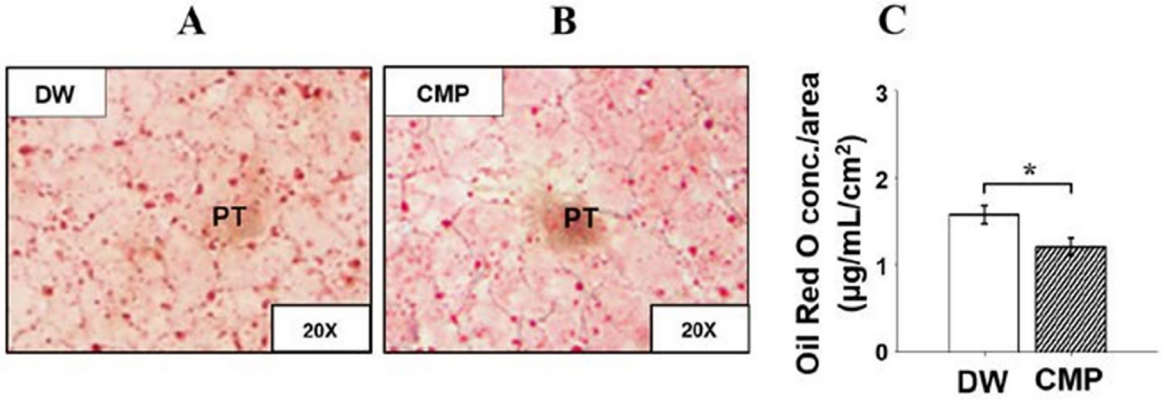


FIGURE 3 - Effects of CMP (1 g/kg) or distilled water (DW) consumption by the middle-aged male rats on liver cell lipid accumulation. (A) Distilled water (DW), (B) CMP and (C) oil red O concentration. Values represent mean ± SEM of 6 experiments. * Significantly lower than that of the distilled water control group, $P < 0.05$. (PT = Portal triad; oil red O staining of liver tissue frozen section, 20 mm thick, 20X magnification).

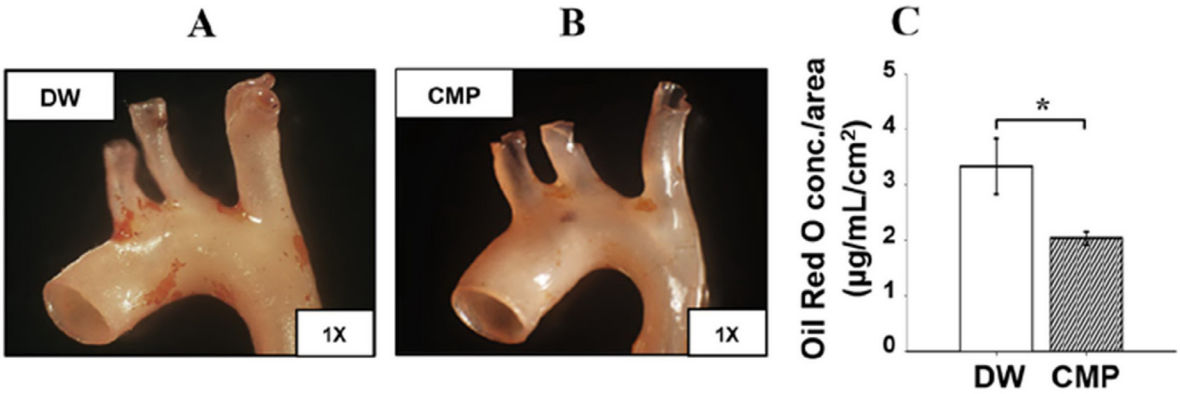


FIGURE 4 - Effects of CMP (1 g/kg) or distilled water (DW) consumption by the middle-aged male rats on aortic arch lipid accumulation. (A) Distilled water (DW), (B) CMP and (C) oil red O concentration. Values represent mean ± SEM of 6 experiments. * Significantly lower than that of the distilled water control group, $P < 0.05$.

SUPPL. TABLE I - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by middle-aged male rats on fasting plasma glucose and lipid profile.

NLAC-MU	Glucose	Triglyceride	Cholesterol	HDL-C	LDL-C	LDL/HDL
normal range						**ratio**
(mg %)	**122.1–180.8**	**61.0–164.0**	**46.0–98.0**	**-**	**-**	**-**
DW	132.2 ± 5.2	78.2 ± 7.9	95.6 ± 2.6	99.0 ± 5.5	25.3 ± 2.0	0.3 ± 0.03
CMP	131.0 ± 5.9	84.2 ± 11.2	90.8 ± 7.7	98.7 ± 5.6	23.3 ± 3.5	0.2 ± 0.05

Note: NLAC-MU = National Laboratory Animal Center Mahidol University.

SUPPL. TABLE II - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by middle-aged male rats on the plasma levels of alkaline phosphatase (Alp), Blood urea nitrogen (BUN) and Creatinine (CREAT).

NLAC-MU	ALP (U/L)	SGOT (U/L)	SGPT (U/L)	BUN (mg %)	CREAT (mg %)
normal range	**46.0 – 92.0**	**111.0 – 225.0**	**25.0 – 64.0**	**10.3 – 23.6**	**0.5 – 0.7**
DW	74.4 ± 7.8	123.9 ± 12.1	80.7 ± 5.6	23.8 ± 2.3	0.6 ± 0.1
CMP	71.4 ± 4.3	120.7 ± 11.4	77.6 ± 3.5	25.7 ± 3.9	0.7 ± 0.2

Note: NLAC-MU = National Laboratory Animal Center Mahidol University.

TABLE I - Effects of CMP (1 g/kg) or distilled water (DW) consumption by middle-aged male rats on complete blood count

NLAC- MU	n	HCT (%)	HGB (g/dL)	MCV (fL)	MCH (pg)	MCHC (%)	WBC ($x10^3$/µL)	Neutrophil (%)	Lymph (%)	Plt ($x10^5$/µL)	N/L ratio
normal range		**33.2 – 46.0**	**13.5 – 17.6**	**47.5 – 54.7**	**17.4 – 26.5**	**34.7 – 51.8**	**3.0 – 7.2**	**-**	**59.0 – 91.0**	**4.9 – 11.3**	**-**
DW	6	46.8 ± 2.7	16.0 ± 0.8	51.6 ± 0.2	17.4 ± 0.2	33.4 ± 0.7	5.5 ± 0.6	68.2 ± 6.1	31.4 ± 6.1	8.6 ± 0.3	2.7 ± 0.6

TABLE I - Effects of CMP (1 g/kg) or distilled water (DW) consumption by middle-aged male rats on complete blood count

NLAC- MU	n	HCT (%)	HGB (g/dL)	MCV (fL)	MCH (pg)	MCHC (%)	WBC (x10^3/µL)	Neutrophil (%)	Lymph (%)	Plt (x10^5/µL)	N/L ratio
CMP	6	46.1 ± 2.3	16.2 ± 0.7	52.9 ± 1.2	18.4 ± 0.4	34.7 ± 0.4	4.4 ± 0.3	63.3 ± 4.8	35.4 ± 4.9	7.4 ± 0.6[a]	2.1 ± 0.4

[a] Significantly lower than the DW control group, $P < 0.05$.
Note: NLAC-MU = National Laboratory Animal Center Mahidol University

TABLE II - Effects of CMP (1 g/kg) or distilled water (DW) consumption by middle-aged male rats on the relative adipose tissue weight (g/100 g body weight)

Treatments	Adipose tissue weight/100 g body weight (% g)				
	Epididymis	Prostate	Mesentery	Retroperitoneal	Subcutaneous
DW	2.48 ± 0.15	0.18 ± 0.03	2.43 ± 0.20	3.23 ± 0.58	7.70 ± 0.68
CMP	2.22 ± 0.06	0.19 ± 0.00	1.78 ± 0.14[a]	1.97 ± 0.29[a]	5.28 ± 0.68[a]

[a] Significantly lower than DW control group, $P < 0.05$.

Suppl. TABLE III - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by middle-aged male rats on the relative adipose tissue accumulation at the internal organs and at the subcutaneous (g/ 100 g body weight) of middle-aged male rats.

Treatments	Organs weight/ 100 g body weight (% g)								
	Heart	Lung	Liver	Kidney	Adrenal gland (mg)	Spleen	Testis	Epididymis	Prostate gl.
DW	0.3 ±	0.3 ±	2.7 ±	0.5 ±	13.4 ±	0.2 ±	0.6 ±	0.3 ±	0.2 ±
	0.01	0.01	0.15	0.02	0.99	0.01	0.01	0.01	0.02
CMP	0.3 ±	0.3 ±	2.7 ±	0.6 ±	14.3 ±	0.2 ±	0.6 ±	0.3 ±	0.2 ±
	0.01	0.01	0.16	0.06	0.71	0.01	0.01	0.01	0.02

Effects of CMP treatment on blood pressure

CMP treatment did not affect the basal arterial blood pressure or heart rate of the anesthetized middle-aged rats when compared to that of the DW control group (Suppl. Table IV).

SUPPL. TABLE IV - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by middle-aged male rats on blood pressure and heart rate in anesthetized middle-aged male rats.

Treatments	Body weight (g)		n	Basal systolic BP	Basal diastolic BP	Mean Arterial Pressure	Basal heart rate
	Initial	Final		(mmHg)	(mmHg)	(mmHg)	(bpm)
DW	481.9 ± 13.1	492.1 ± 8.4	6	134.2 ± 5.4	109.2 ± 4.6	120.8 ± 2.0	425.0 ± 11.2
CMP	488.0 ± 7.9	505.1 ± 7.2	6	136.7 ± 9.6	109.6 ± 8.0	118.6 ± 8.5	428.3 ± 10.9

Effects of CMP treatment on vascular functions

Effect on contraction and relaxation of the thoracic aorta

No significant difference was found in the contractile responsiveness to phenylephrine of the thoracic aortic rings obtained from the CMP-treatment group compared to that of the DW control group. This situation persisted in the presence of L-NA, and even when PAG was also added (Figure 5 and Suppl. Table V).

The relative relaxation to acetylcholine of endothelium-intact aortic rings precontracted with phenylephrine was not different between the CMP- and the DW-treatment group. PAG caused lower relaxation of the aortic rings of both groups to the same extent (Figure 6A). Similarly, vasorelaxation to sodium nitroprusside of the thoracic aortic rings of the CMP-treatment group was similar to that of the DW control group, both in the presence and absence of PAG (Figure 6B). In the case of GTN, the vasorelaxation of the endothelium-intact aortic rings in the presence of L-NA of the CMP-treated rats was greater than that of the DW control group (Figure 6C and Suppl. Table III) and this effect was not modified by PAG (Figure 6D). A low concentration of ODQ (1μM) was able to decrease the relaxation of the aortic ring to GTN of both groups, but the relaxation of the CMP-treated group was still higher than that of the DW control group. However, when the concentration of ODQ was increased to 10μM, almost complete inhibition of the GTN induced relaxation was noted (Figure 7).

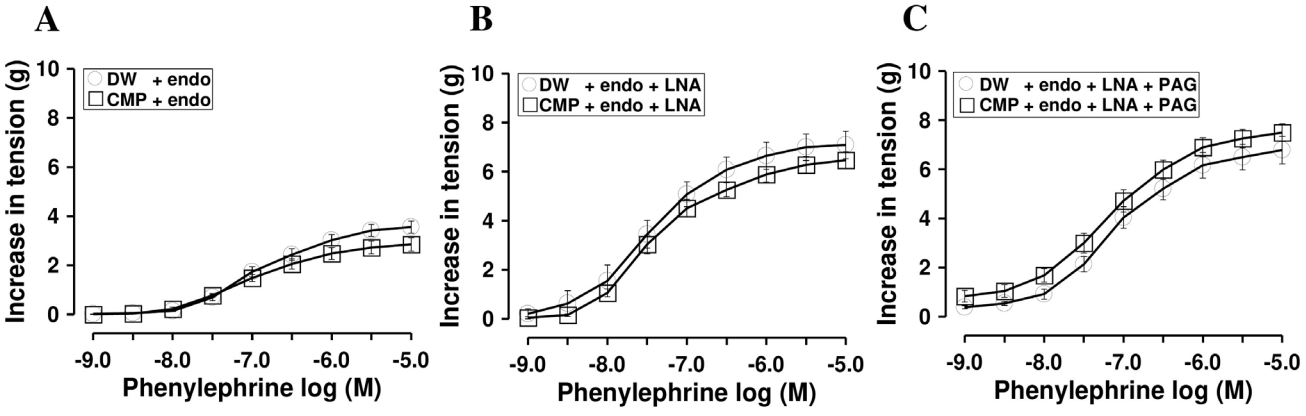


FIGURE 5 - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by the middle-aged male rats on contractile response to phenylephrine of endothelium-intact (endo, A), endothelium-intact with L-NA (B), endothelium-intact in the present of L-NA and PAG thoracic aorta (C). Values represent mean ± SEM; n = 6.

Suppl. TABLE V - Effects of CMP (1 g/kg) or distilled water (DW) consumption by middle-aged male rats on EC_{50} and E_{max} values of phenylephrine on contraction of the endothelial-intact (Endo) or denuded (No endo) aortic rings in the absence or presence of N-nitro-L-arginine (L-NA) and/ or DL-propargylglycine (PAG).

	EC_{50} (nM) : 95% confidential limit		E_{max} (g)	
	DW	**CMP**	**DW**	**CMP**
Phenylephrine				
Endo	14.0 (7.8-25.2)	25.2 (16.0-39.7)	3.6 ± 0.3	2.9 ± 0.3
Endo+L-NA	19.3 (13.3-31.2)	24.0 (15.7-37.0)	7.1 ± 0.6	6.5 ± 0.4
Endo+L-NA+PAG	67.6 (34.6-98.0)	30.1 (19.2-49.7)	6.8 ± 0.6	7.5 ± 0.5
No endo	26.2 (8.6-79.6)	33.7 (14.7-77.0)	4.7 ± 0.3	4.8 ± 0.2
No endo+L-NA	21.1 (9.2-48.4)	36.4 (15.6-84.9)	6.7 ± 0.4	6.9 ± 0.1
No endo+L-NA+PAG	21.7 (10.7-44.2)	11.3 (6.3-20.3)	8.3 ± 0.6	8.2 ± 0.2

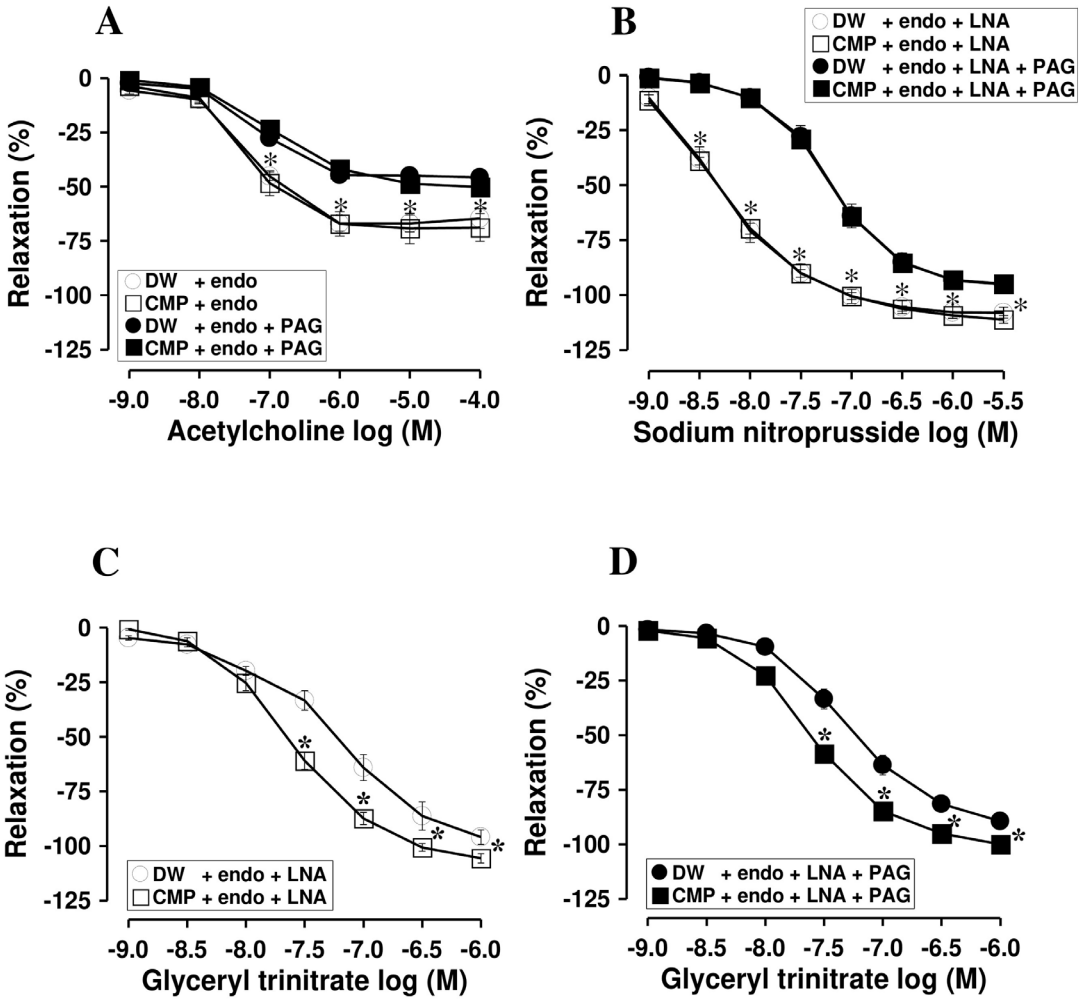


FIGURE 6 - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by the middle-aged male rats on relaxation of the endothelium-intact thoracic aortic ring precontracted with phenylephrine to acetylcholine (A), to sodium nitroprusside (B), to glyceryl trinitrate (C) or to glyceryl trinitrate in the presence of L-NA and PAG (D). Values represent mean ± SEM; n = 6. * Significantly lower than the DW control group, $P < 0.05$.

TABLE III - Effects of CMP (1 g/kg) or distilled water (DW) consumption by middle-aged male rats on EC_{50} and E_{max} values of acetylcholine, sodium nitroprusside or glyceryl trinitrate on relaxation of the endothelial-intact (Endo) aortic rings in the absence or presence of N-nitro-L-arginine (L-NA) and/or DL-propargylglycine (PAG)

	EC_{50} (nM) : 95% confidential limit		E_{max} (g)	
	DW	**CMP**	**DW**	**CMP**
Acetylcholine				
Endo	53.6 (5.1-268.0)	62.7 (5.3-297.5)	64.6 ± 4.6	68.8 ± 6.3
Endo+PAG	72.0 (9.8-329.0)	128.9 (10.0-366.9)	45.7 ± 3.0	50.2 ± 3.2
Sodium nitroprusside				
Endo	13.5 (9.9-18.2)	11.9 (10.8-13.1)	119.0 ± 4.7	125.4 ± 2.9
Endo+L-NA	3.7 (2.6-5.2)	3.3 (2.2-5.1)	108.1 ± 2.5	111.2 ± 1.8
Endo+L-NA+PAG	61.3(55.4-67.9)	59.8(55.8-64.1)	95.1 ± 2.9	94.9 ± 1.8
Glyceryl trinitrate				
Endo	64.4 (32.6-117.5)	35.0 (29.1-42.0)	68.1 ± 4.1	93.8 ± 4.1[a]
Endo+L-NA	48.7 (28.1-84.3)	27.4 (11.3-66.7)	95.9 ± 3.4	105.6 ± 2.1[a]
Endo+L-NA+PAG	50.5 (34.8-73.3)	27.4 (9.6-78.6)	89.4 ± 2.7	100.0 ± 1.3[a]

Significantly higher than DW control group, $P < 0.05$.

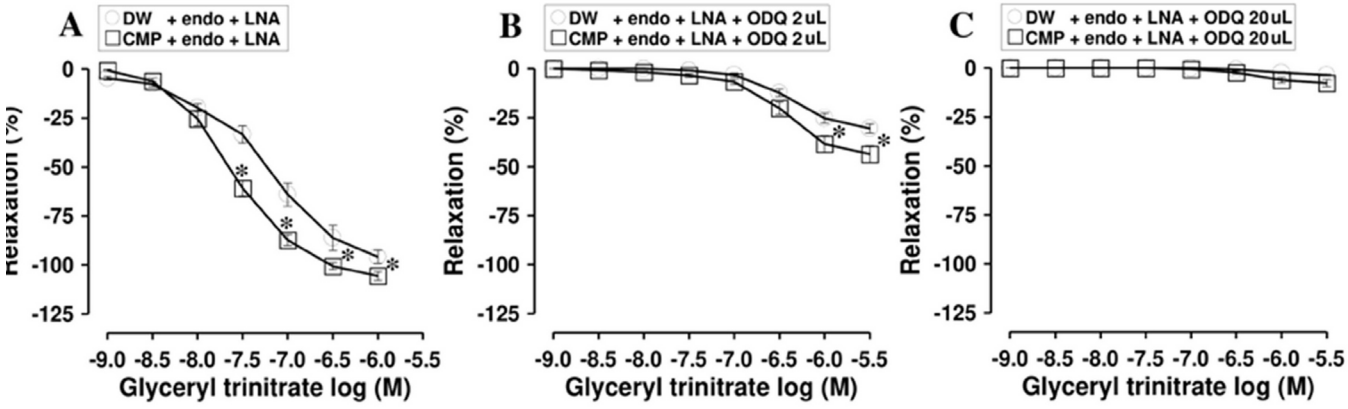


FIGURE 7 - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by the middle-aged male rats on relaxation of the endothelium-intact thoracic aortic ring precontracted with phenylephrine to glyceryl trinitrate in the presence of (A) L-NA, (B) L-NA and 1 μM ODQ and (C) L-NA and 10 μM ODQ. Values represent mean ± SEM; n = 6. * Significantly lower than the DW control group, $P < 0.05$.

eNOS, CSE and sGC western blot analysis

Western blot results were compared between CMP-treated and DW control group. For each group, the intensity of the protein concerned was divided by the intensity of actin for all of four independent tissue samples. The results showed no significantly different in eNOS, CSE and sGC expression between the CMP-treated and DW control group (Figure 8).

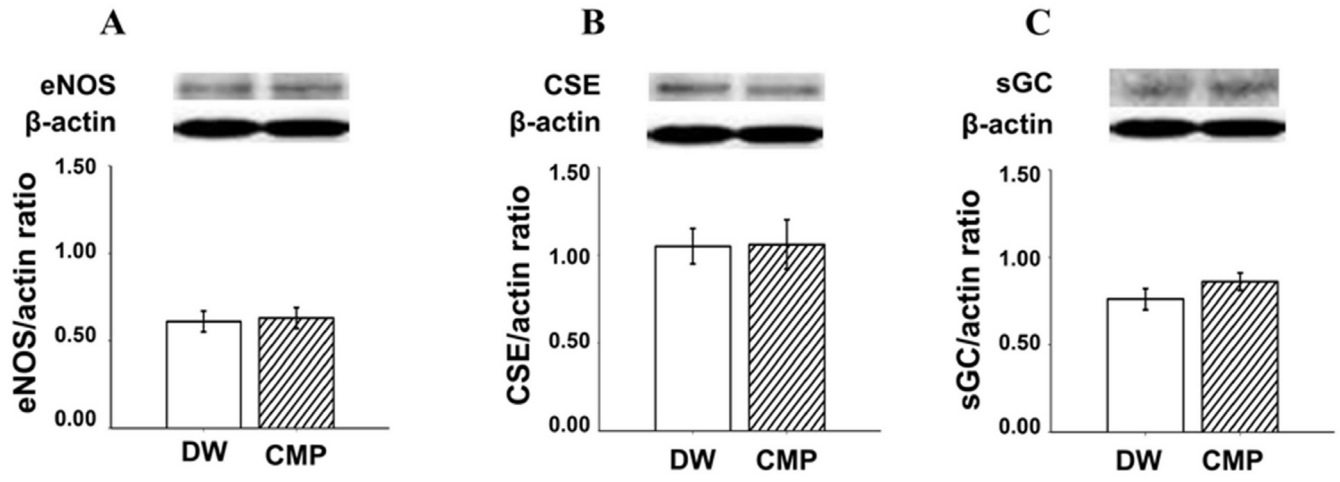


FIGURE 8 - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by the middle-aged male rats on eNOS protein expression (A), CSE protein expression (B), or sGC protein expression (C) of the thoracic aorta. For each blot, β-actin expression is shown as a loading control. Values represent mean ± SEM; n = 4.

Aortic ring cGMP levels

The basal cGMP level of the thoracic rings in the presence of L-NA obtained from the CMP-treated group was not significantly higher than that of the DW control group. In the presence of GTN, the blood vessel cGMP levels were significantly increased in both groups and that of the CMP-treated group was higher than that of the DW control group (Figure 9).

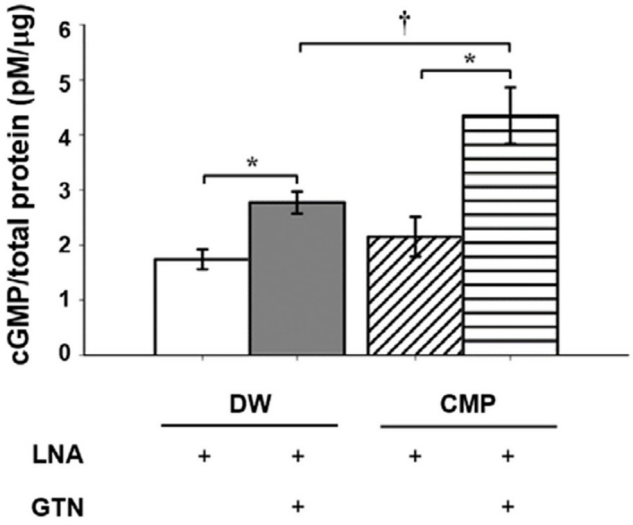


FIGURE 9 - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by the middle-aged male rats on blood vessel cGMP level. * Significantly higher than their corresponding control, and † significantly higher than the DW control group, $P < 0.05$.

Effects of GTN and diadzin on blood vessel nitric oxide concentration

In the presence of L-NA, GTN stimulated higher release of NO from the aortic rings of the CMP-treated rats than that of the DW control group. Diadzin inhibited the NO production of the aortic vessel from both the CMP- and DW-treated rats to the same extent, thus the aortic vessel NO concentration of the CMP-treated rats was still higher than that of the DW control group (Figure 10).

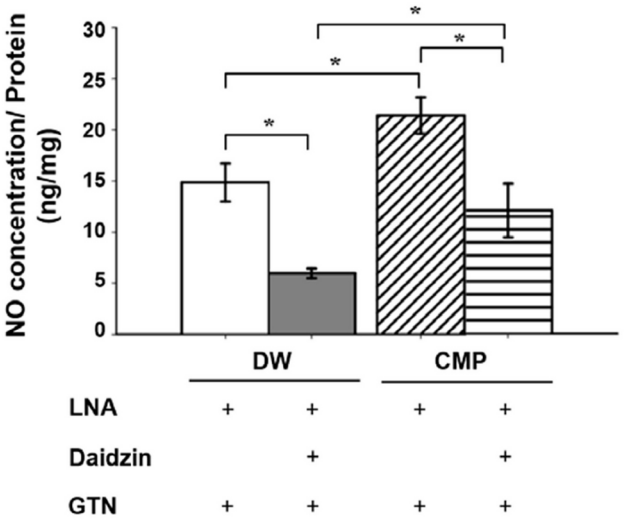


FIGURE 10 - Effects of CMP (1 g/kg) or distilled water (DW, control) consumption by the middle-aged male rats on NO concentration/protein (ng/mg) in aortic rings. * Significantly lower than their corresponding control, and † significantly higher than the DW control group, $P < 0.05$.

DISCUSSION

The present study demonstrates that CMP consumption caused decreased aortic arch, body and internal organ fat accumulation, as well as liver cell lipid accumulation when compared to that of the distilled water control group which is the vehicle for CMP dissolution. The mechanism responsible for this might be a result of the CMP inhibiting the lymphatic lipid transport in the thoracic lymph duct, as was shown by Matsuoka *et al*. (2014). This result is analogous to that reported by Padmakumaran Nair, Rajamohan, Kurup (1998), who found that the consumption of coconut kernel protein by young adult rats caused lower lipogenesis in the liver, and that of Salil, Rajamohan (2001), who found a reduction of hyperlipidemia and a peroxidative effect in rats fed a high fat-cholesterol diet.

CMP consumption did not alter the basal blood pressure nor the heart rate, but caused a beneficial change in the blood vessels, with increased relaxation of the aortic ring when exposed to GTN, but not to sodium nitroprusside, a direct nitric oxide donor. This effect was not found in a previous study by this research group of the effect of dried fresh coconut-milk consumption by middle-aged male rats (Naphatthalung *et al*., 2018). The reason for this might be the different amounts of coconut protein consumed by the rats in each study. In the present study the rats were gavaged 1 g/kg coconut protein which was consistent with the recent recommendation for protein intake for individuals with minimal physical activity (Wu, 2016). However, in the previous study the amount of protein in the dried fresh coconut milk was about 0.58 g/kg (dried fresh coconut milk contains 19.35 %, thus a dosage of 3 g/kg coconut milk would be 3 x 0.1935 = 0.58 g of protein) which is about half that in the present study. Therefore, the amount of the active component in the protein gavaged to the rats in the previous study might not reach the threshold strength required to produce such activity. However, further study would be needed to clarify this possibility by using different dosages as well as identifying the active component(s) of the coconut protein.

GTN is a vasodilator drug that has been used clinically since the late nineteenth century for the treatment of angina pectoris, congestive heart failure and myocardial infarction, but the mode of action of the drug is still a matter of debate (Bonini *et al*., 2008; Mayer, Beretta, 2008). To date, however, the evidence suggests that the molecular mechanisms of GTN firstly need it to be bio-transformed to NO, which results in vascular cGMP accumulation and vasorelaxation (Diamond, Blisard, 1976; Kawamoto *et al*., 1990), and mitochondrial aldehyde dehydrogenase-2 (ALDH-2) might be one of the enzymes responsible in the pathway (Kollau *et al*., 2005; Mayer, Beretta, 2008; Opelt *et al*., 2016; 2018). Meanwhile, Bonini *et al*. (2008) demonstrated that GTN triggers eNOS to generate NO, while Artz *et al*. (2001) reported that GTN behaves as a partial agonist with respect to sGC activation. In the

present study, therefore, we investigated whether the increased vasorelaxant activity of the GTN might involve (1) increased sGC activity, (2) the up-regulation of blood vessel sGC protein expression, or (3) increased ALDH-2 activity involving the biotransformation of the GTN to NO. In order to elucidate these possibilities, classical pharmacologic methods using different inhibitors (L-NA for eNOS, ODQ for sGC and diadzin for ALDH-2) were used to test the relaxation responsiveness of the aortic rings to GTN. In order to prevent any disturbance from NO, all the experiments were performed in the presence of L-NA to inhibit the eNOS activity. As shown in the Results section, only a high concentration of ODQ abolished the relaxation of the aortic rings of both groups, while a low concentration of ODQ was only able to partially inhibit relaxation in both groups, but to the same extent. Therefore, the maximum relaxation of the CMP-treated group was still higher than that of the DW control group. This result suggests that CMP consumption might cause increased sGC activity. Consistent with this, the basal blood vessel cGMP level was not different between the CMP-treated group and the DW control group, but GTN caused a higher increase in the blood vessel cGMP level of the CMP-treated group than that of the DW control group. However, it is unlikely that the increased cGMP level was due to the up-regulation of blood vessel sGC-protein expression, since sGC expression measured by western blot analysis was not found to be different between the CMP-treated group and the DW control group. Another possible explanation of the higher cGMP level caused by GTN stimulation might be due to increased activity of the enzyme, ALDH-2 transforming the GTN to NO. To investigate this possibility, experiments were conducted in the presence of diadzin, a specific ALDH-2 inhibitor, and the NO concentration of the blood vessels was measured in the presence of GTN, with the expectation that if the ALDH-2 had increased, the NO concentration of the CMP group would increase more in the experimental group than in the DW control group with the same concentration of diadzin. Accordingly, it was found that the NO concentration of the CMP-treatment group was higher than the DW control group after partial inhibition by diadzin, indicating that CMP consumption might cause increased ALDH-2 activity to convert GTN to NO. However, further specific experiments based on directly measuring blood vessel ALDH-2 activity would need to be conducted to confirm this.

CMP consumption did not affect the relaxation of the aortic ring to acetylcholine compared to that of the DW control group, indicating that consumption of CMP did not affect the basal- and muscarinic-stimulated release of the endothelial NO production of the aortic blood vessel. This is consistent with the finding that eNOS protein expression did not increase. However, this finding is different from that reported by Salil, Nevin, Rajamohan (2011) who claimed that high arginine-coconut protein consumption by rats produced antidiabetic activity, perhaps via the arginine-NO pathway, leading to pancreatic beta cell regeneration in rats suffering from alloaxan- and streptozotocin-induced diabetes. The reason for this might be due to differences in the amounts of the amino acid constituents in different coconut protein compositions, especially that of the amino acid, arginine. In the present study the crude coconut protein used was isolated directly from dried fresh coconut milk, and contained about 7% arginine (Figure 1B), whereas Salil, Nevin, Rajamohan (2011) used a saline globulin protein fraction isolated from petroleum defatted coconut kernel contained which contained 18.2% the arginine.

In our previous study, it was found that coconut milk consumption caused an increase in blood vessel CSE expression, resulting in increased H_2S, which attenuated the vasoconstriction of the aortic rings to phenylephrine. In order to reveal whether CMP might be responsible for this effect, experiments were performed on the aortic rings of both the CMP- and DW-treated groups. It was found that PAG significantly inhibited the contraction of the aortic rings to phenylephrine, and the relaxation to acetylcholine, GTN or sodium nitroprusside was the same for the CMP- and DW-treated rats, indicating that CMP consumption did not affect blood vessel H_2S production, and this was confirmed by the finding that the blood vessel CSE expression was not different between the CMP- and the DW-treated rats.

CONCLUSION

The present study demonstrated that consumption of CMP by middle-aged male rats caused decreased body fat-, liver cell- and aortic arch lipid accumulation, with

a decrease in the platelet count but had no effect on any of the other blood biochemistry parameters. Although CMP did not affect the basal blood pressure and heart rate, it caused potentiated relaxation of the aortic rings to GTN which is beneficial in prolonging or preventing GTN tolerance which normally occurs after long-term GTN treatment in humans (Jabs *et al*., 2015; Münzel, Daiber, Gori, 2011; 2013). Thus, CMP is a novel protein that should be developed as a health-food ingredient to prevent or slow down the development of cardiovascular disease in human, especially from middle-age onwards. However, further a study would need to be conducted to identify the active component(s) of crude CMP.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund, Thailand and Graduate School, Prince of Songkla University, Hat-Yai, Thailand. The authors thank Miss. Srisurat Duangsai for her technical assistance and Prof. Michael J. Mulvany, Aarhus University, Denmark, for his valuable comments and editing the manuscript. The authors declare no conflicts of interest. CJ designed the research, conducted some experiments and wrote the manuscript, JN, PC and SY conduct the research and analyzed the data, KK assisted with the western blot experiment and NR assisted with the oil red O tissue staining technique. All authors read and approved the final version of the manuscript.

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Received for publication on 26th June 2020
Accepted for publication on 05th April 2021