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Antihypertensive effect of MCHF in L-NAME induced hypertensive rats

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Abstract

Moa Chub's formulation (MCHF), a traditional medicinal formulation, is used in Thailand for treatment of hypertension. It is composed of 7 kinds of medical herbs, *Cyperusrotundus, Tinosporacrispa, Tinosporacordifolia, Acmellaoleracea, Syzygiumaromaticum, Piper chaba, and Zingiberofficinale.* This study aimed to determine the effect of MCHF on blood pressure by investigating nitric oxide (NO) and lipid peroxidation in N-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats. Rats were gavaged with MCHF (100, 200 and 400 mg/kgBW/day) and fed with L-NAME in drinking water for 21 days. Non- invasive tail cuff was used to monitor blood pressure every 7 days. At the end of trial, blood sample was collected and plasma nitric oxide and lipid peroxidation were measured. In addition, the cytotoxicity of MCHF was evaluated in human hepatoma HepG2 cell line. Cell viability of HepG2 cells was measured by MTT assay. The results showed that administration of MCHF extract at dose of 200 and 400 mg/kgBW/day significantly decreased a rise of systolic blood pressure in hypertensive rats induced by L-NAME on the 21st day. Moreover, MCHF significantly increased the serum level of NO and reduced the level of MDA compared to the group that was treated with only L-NAME. The high doses of MCHF may lead to increased risk of hepatotoxicity. These results suggested that MCHF has the potential ability to be used as herbal remedy to treat hypertension. However, further studies are needed to explore the mechanism of antihypertensive effect of MCHF in detail.

Keywords**:** *antihypertensive, hepatotoxicity, lipid peroxidation, Moa Chub's formulation, MCHF, L-NAME, nitric oxide*

1. Introduction

Hypertension is one of the most important factors associated with the development of vascular diseases. Hypertension is common progressive disorder leading to numerous chronic diseases such as cardiovascular disease, stroke, renal disease and accounts for about 50% of cardiovascular disease worldwide (Joffres et al., 2013; Kearney et al., 2005). Nitric oxide (NO) synthesis and release by endothelial cells are important vascular relaxation effects that contribute to the modulation of vascular tone (Katsumi, Nishikawa, and Hashida, 2007; Mori et al., 2006). Chronic inhibition of NO produces volume-dependent evaluation of blood pressure and its physiological and pathological characteristics resemble essential hypertension. In addition, it is well established that acute inhibition of nitric oxide biosynthesis by in vivo administration of N-nitro-L arginine methyl ester hydrochloride (L-NAME), an L-arginine analogue, leads to arterial hypertension and vasoconstriction (Attia et al., 2001; Ribeiro, Antunes, De Nucci, Lovisolo, & Zatz, 1992). Evidence suggests that NO plays a major role in regulating blood pressure and that impaired NO bioactivity is an important component of hypertension. Mice with disruption of the gene for endothelial NO synthase have elevated blood pressure levels, suggesting that a genetic component might link between impaired NO bioactivity and hypertension (Shesely et al., 1996). Clinical studies have shown that patients with hypertension have a blunted vasodilation of arterial response to infusion of endothelium-dependent vasodilators and that inhibition of NO raises blood pressure. Impaired NO bioactivity is also implicated in arterial stiffness, a major mechanism of systolic hypertension (Panza, Casino, Kilcoyne, & Quyyumi, 1993).

It was reported earlier that free radicals may contribute to the pathogenesis of human essential hypertension (Kumar & Das, 1993; Nakazono et al., 1991). Many studies indicate that oxidative stress is involved in the pathogenesis of arterial hypertension in genetic animal models and in secondary forms of arterial hypertension (Vaziri, Lin, Farmand, & Sindhu, 2003). On the

other hand, several researchers have proposed that oxidative stress contributes to the generation or maintenance of hypertension via inactivation of NO (Ortiz, Manriquez, Romero, & Juncos, 2001). Hypertension produced by NO synthesis inhibition is associated with increased oxidative stress via inhibiting its vasodilatory and natriuretic actions and via non enzymatic generation of vasoconstrictor isoprostanes from arachidonic acid peroxidation, with direct vasopressor and antinatriuretic effects (Duarte et al., 2002; Takahashi et al., 1992). Hypertensive patients show increased levels of plasma superoxide, hydrogen peroxide and lipid peroxide (Kumar & Das, 1993; Lacy, O'connor, & Schmid-Schönbein, 1998; Tse et al., 1994). Moreover, in vessels from spontaneously hypertensive rats (SHR) and essential hypertensive rats, enhanced endothelial superoxide anion production has been described and this effect has been related to the impairment of endothelium-dependent relaxation (Grunfeld et al., 1995; Jameson et al., 1993; Suzuki, Swei, Zweifach, & Schmid-Schönbein, 1995).

Herbal medicines are very prevalent, being used in the developing countries for a primary health care (Khare, 2008). Moa Chub's formulation (MCHF), Thai formulated herbs for antihypertension, has long been used to relieve high blood pressure. It consists of seven types of herbs, which are *Cyperusrotundus, Tinosporacrispa, Tinosporacordifolia, Acmellaoleracea, Syzygiumaromaticum, Piper chaba, and Zingiberofficinale*. An in vivo study indicated that MCHF at the oral dose of 5 g/kgBW, which is 60 times higher than dose in humans, caused no abnormalities. In addition, no animal died within 24 hours and all animals survived until 14 days after herb administration. The blood biochemical tests to evaluate renal and liver functions showed that the levels of creatinine, protein, albumin and bilirubin did not differ between treated group and normal group. The LD_{50} of MCHF is higher than 5,000 mg/kgBW, which is classified as a substance with no acute toxicity by US EPA Categories (Godsan & Choosongsang, 2012). In sub-chronic toxicity tests, MCHF equivalent dose in humans (400 mg/kgBW) did not cause any abnormalities in animal model. However, there has never been any study reporting about antihypertensive effect of this formulation. Therefore, we have explored the antihypertensive effect of MCHF.

2. Objectives

This study aimed to investigate the antihypertensive effect of MCHF in L-NAME induced hypertension in rats. In addition, we determined the effect of MCHF on serum nitric oxide level, lipid peroxidation and hepatocellular toxicity.

3. Materials and methods

3.1 Preparation of extracts

MCHF were extracted with hot water accordingly to the same manner as used in human. MCHF were crushed into powder and decocted with boiling water three times for 1 hour. The total decoction was mixed and filtered to remove the residue. Spray drying process was carried out to remove any residual trace of extraction solvent in the extract. Each gram of MCHF is equivalent to 5 g of dried starting materials. The extraction powder of MCHF was stored at 4ºC until used. Animal doses of MCHF were calculated from human doses based on body surface area (BSA) (Nair & Jacob, 2016; Shin, Seol, & Son, 2010). MCHF extract 100, 200 and 400 mg/kgBW/day were used in the experiment.

3.2 Animals

Male Wistar rats weighing 200 ± 30 g were used for the study. Rats were obtained from the National Laboratory Animal Center, Salaya, Mahidol University, Nakorn Pathom, Thailand. They were housed in animal room with a constant temperature maintained at 25 ± 2ºC under a 12-hour light and dark cycle and they had free access to water and food. Rats were randomly divided into five groups of 6 animals each: control (distilled water), L-NAME 50 mg/kgBW/day, L-NAME 50 mg/kgBW/day plus MCHF extract 100, 200 and 400 mg/kgBW/day. Rats received L-NAME in their drinking water in order to induce hypertension. MCHF has been tested for its blood pressure lowering activity in rats render hypertensive by L-NAME for 21 days. Blood samples were collected at the end of the experiment. Plasma was separated for assessing nitric oxide and lipid peroxidation. The experimental protocol was approved by the Ethics Committee for Animal Research, Rangsit University (RSEC 03/2559).

3.3 Measurement of blood pressure.

The systolic blood pressure (SBP) of all animals was measured weekly by tail-cuff method

using the MLT125/R NIBP System in conjunction with a PowerLab system (AD instruments, Australia). In brief, conscious rats were placed in restrainer tube leaving the tail outside and adjusted to the position where the animal has limited movement. The tail of each rat was placed inside the tail cuff, and the cuff was automatically inflated and released. The rat's tail was warmed by a lamp for 10 minutes before every blood pressure measurement to easily detect the pulsation of the tail artery. The blood pressure was recorded as the mean value from the three measurements with 15-minute intervals.

3.4 Assay of nitric oxide (NO)

The concentration of plasma NO was measured by using enzymatic conversion method with some modifications (Green et al., 1982; Sun, Zhang, Broderick, & Fein, 2003). Briefly, plasma samples were mixed with Griss reagent 1% N-(1-naphthyl)ethylene-diaminedihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The absorbance of samples at 548 nm was measured on microplate reader (Benchmark Plus, Biorad®). The amount of nitrite in the samples was estimated from the calibration curve using sodium nitrile ($NaNO_2$) standard.

3.5 Assay of malondialdehyde

Lipid peroxidation (LPO) level was measured by the method of Buege and Aust (Buege & Aust, 1978) and evaluated by measuring the MDA concentration, which is the end product of LPO. The plasma was precipitated with trichloracetic acid. After centrifugation (3000×g, 15 minutes), the supernatant was mixed with thiobarbituric acid (TBA) reagent and the mixture was kept at 100ºC for 15 minutes. The level of LPO was measured based on the formation of TBA reactive substance (TBARS) to produce a red colored complex with a peak absorbance at 535 nm by using a spectrophotometer. The standard curve was generated at different concentrations of 1,1,3,3-tetraethoxypropane (TEP).

3.6 Hepatotoxicity

The human hepatocellular carcinoma cell line HepG2 was used as a model (Snopov, Teryukova, Sakhenberg, Teplyashina, & Nasyrova, 2017; Donato, Jover, & Gomez-Lechon, 2013; Yeon, Na, & Park, 2010). The HepG2 cells cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco/Life Technologies, Stockholm, Sweden).

3.6.1 Determination of hepatotoxicity

Cell viability of HepG2 cells was determined by MTT assay. Following the treatment, cells were incubated with MTT solution (5.0 mg/mL in PBS) at 37°C with 5% CO_2 for 4 hours. Then, the solution was replaced with DMSO to solubilize the formazan product. The intensity of the formazan product was measured at 570 nm by using a microplate reader. Cell viability was expressed as the percentage calculated from the optical density of treated cells relative to the controlled cells. The percentage of cytotoxicity was calculated by subtracting the percent viability of treatment group from the percent viability of control group. The CD_{50} (median cytotoxic dose) was calculated.

3.6.2 Acute hepatotoxicity

Cells were seeded at the density of 4×10^5 cells/mL onto 24-well plate overnight. After that, they were treated with MCHF at the concentrations of 0 - 50 mg/mL for 24 hours. Then, cell viability was measured by MTT assay. The percentage of cell viability and CD_{50} were calculated as described above. The non-toxic concentrations were used for subacute hepatotoxicity determination.

3.6.3 Subacute hepatotoxicity

Cells were seeded into 6-well plate at the initial plating density of 2×10^5 cells/mL. Cells were allowed to adhere to the surface of the plates for 4 hours then they were treated with MCHF at the non-toxic concentrations. The treated cells were sub-cultured and exposed to the formulation every two days. The cells were subsequently collected on 7^{th} day for cell viability determination by MTT assay. The percentage of cell viability was calculated as described above.

3.7 Statistical analysis

All the data were expressed as mean ± SEM (standard error of the means). Results were statistically analyzed by one- way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. $p<0.05$ were considered as significant difference.

4. Results

4.1 Effect of MCHF on systolic blood pressure

In this study, blood pressure was recorded every 7 days for 21 days of treatment. At the beginning of the study, average baseline values of systemic blood pressure (SBP) among all groups of rats were not significantly different. In the control group, the SBP did not change throughout the 21 days of the experiment (Figure 1). The daily administration of L-NAME in the dose of 50 mg/kgBW/day for 21 days caused a progressive increase in SBP compared to SBP levels in control rats (127.50 ± 3.61, 133.14 ± 3.63, 149.09 ± 4.93, 154.97 ± 4.20). The results showed that administration of MCHF extract at the dose of 200 and 400 mg/kgBW/day significantly decreased a rise of SBP in hypertensive rats induced by L-NAME on the 21st day. Blood pressure on the 21st day of rats treated with L-NAME, L-NAME plus MCHF 100, 200 and 400 mg/kgBW/day were 154.97 ± 4.20, 155.79 ± 11.88, 138.98 ± 10.52, 137.63 ± 5.64 mmHg, respectively (Figure 1).

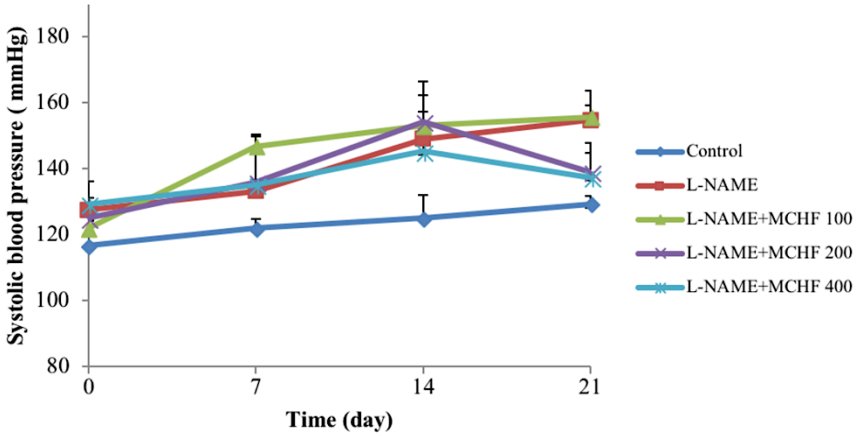

Figure 1 Effect of MCHF on systolic blood pressure (SBP) during L-NAME administration for 21 days in hypertensive rats. Results were expressed as mean ± SEM. (n=6/group). All of the values were statistically analyzed by ANOVA: **p*<0.05 when compared with L-NAME group.

4.2 Effect of MCHF on nitric oxide (NO) production

The NO level decreased significantly in L-NAME administrated rats when compared to control. Plasma NO level of control group, groups treated with L-NAME and L-NAME plus MCHF 100, 200 and 400 mg/kgBW/day for 21 days were 1.44 ± 0.21, 5.48 ± 0.34, 5.87 ± 0.31, 4.20 ± 0.66 and 5.29 ± 0.36 µmol/L, respectively (Figure 2). The results showed that the decreasing of NO level by L-NAME administration was significantly reversed by administration with MCHF when compared to the group treated with only L-NAME. However, there was no significant difference in the plasma NO concentrations between each dose of MCHF.

4.3 Effect of MCHF on lipid peroxidation

The data showed that serum MDA significantly increased in L-NAME-induced hypertensive rats. Serum MDA level decreased greatly in MCHF treated rats compared with control. Plasma MDA levels of control group, groups treated with L-NAME and L-NAME plus MCHF 100, 200 and 400 mg/kgBW/day for 21 days were 2.64 ± 0.19, 7.07 ± 1.72, 2.10 ± 0.26, 3.20 ± 0.56 and 2.34 ± 028 µmol/L, respectively (Figure 3).

4.4 Effect of MCHF on hepatotoxicity

To determine the CD_{50} of this formulation, HepG2 cells were treated with MCHF at the concentrations of 0 - 50 mg/mL for 24 hours and cell viability was assessed by MTT assay. Figure 4A shows that the formulation significantly decreased cell viability at the concentration of 5 mg/mL. Therefore, the concentrations of the formulation at 0 – 0.5 mg/mL, which had cell viability more than 90%, were used for continuous treatment. The CD_{50} of this formulation was 5.621 mg/mL (95%CI = 4.071-7.555 mg/mL). Since

patients have to continuously take MCHF every day to control their blood pressure, the effect on HepG2 cells viability with long term exposure was investigated by having the cells continuously treated with the formulation at the concentrations of 0, 0.05 and 0.5 mg/mL for 7 days and determining cell viability by MTT assay. As shown in Figure 4B, the viability of HepG2 cells was significantly decreased by the formulation at the concentration of 0.5 mg/mL.

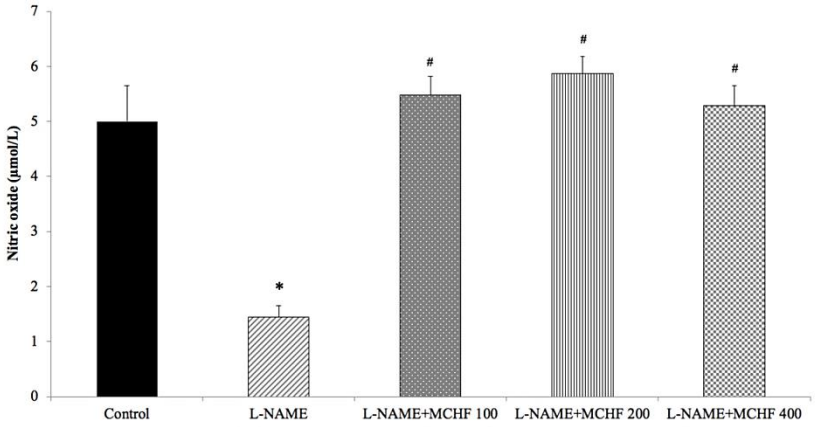

Figure 2 Effect of MCHF on serum NO. Results were expressed as mean ± SEM. (n=6/group). All of the values were statistically analyzed by ANOVA: *p<0.05 when compared with the control. #p<0.05 when compared with L-NAME group.

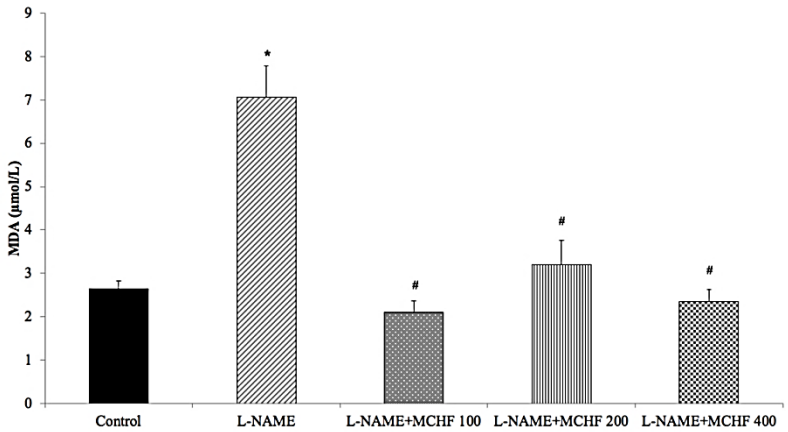

Figure 3 Effect of MCHF on serum MDA levels. Results were expressed as mean ± SEM. (n=6/group). All of the values were statistically analyzed by ANOVA: *p<0.05 when compared with the control. #p<0.05 when compared with L-NAME group.

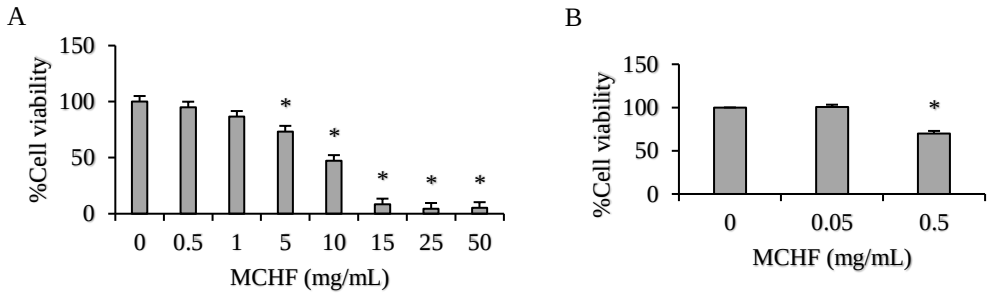

Figure 4 Effect of MCHF on HepG2 cell viability for 1 day (A) and 7 days (B). Values were means of the three independent triplicate samples ± SEM. *p<0.05 versus non-treated control.

5. Discussion

In the present study, we found that L-NAME caused a significant elevation in SBP during the 21 days of treatment, and this hypertension was attenuated by administration of MCHF (Figure 1). L-NAME, a potent NOS inhibitor, induced hypertension in rats from NO deficiency-induced endothelial dysfunction and increase of blood pressure (Doggrell & Brown, 1998). In addition, previous studies have suggested that NO deficiency leads to increased accumulation of superoxide anion in biological tissue which causes oxidative stress in the body with is in turn involved in pathophysiological of many forms of hypertension (Kopkan & Majid, 2005). The concomitant use of MCHF extract in the dose of 100 mg/kgBW/day was not effective in suppressing the rise in blood pressure induced by L-NAME. Whereas, MCHF at the dose of 200 and 400 mg/kg/day caused a reduction in the rise of blood pressure in L-NAME treated rats at 21 days. Thus, MCHF was found to be herbal formulation with potential ability to treat hypertension.

L-NAME induced hypertension is associated with an inactivation of NO system and increase of lipid peroxidation (Figures 2 and 3). Nitric oxide, a free radical, is highly unstable and gets converted into an equimolar ratio of its stable metabolites, nitrite/nitrate. Plasma levels of nitrite/nitrate, which are the end-products of NO, have been frequently determined in order to investigate the possible involvement of NO with the pathogenesis of hypertension. In L-NAME induced hypertensive rats, there was a decrease in NO level as compared to control (Figure 2). NO synthesis and release by endothelial cells are important vascular relaxation effects that contribute to the modulation of vascular tone (Mori et al., 2006). The decrease in NO level produces vasoconstriction of arteries which results in increased cardiac output hence increase blood pressure. On supplementation with MCHF, plasma NO level increased significantly compared to the group treated with only L-NAME (Figure 2). It showed that MCHF may protect cells from NO destruction which leads to decreased blood pressure. However, there were no significant differences in the plasma NO concentrations between each dose of MCHF treated group.

Furthermore, there is clear evidence indicating that oxidative stress plays an important role in the pathophysiology of cardiovascular diseases, mainly arterial hypertension (Ülker, McKeown, & Bayraktutan, 2003). Increased levels of oxidative stress markers, plasma malondialdehyde (MDA) was observed in L-NAME induced hypertensive rats (Bunbupha, Pakdeechote, Kukongviriyapan, Prachaney, & Kukongviriyapan, 2014; Nakmareong et al., 2012). To evaluate the protective role of MCHF against hypertension-associated oxidative stress, lipid peroxidation products (TBARS) in plasma was examined. In this study, L-NAME treatment showed an increase in the levels of TBARS in plasma. Increased lipid peroxidation appeared to be the initial stage of the tissue making it more susceptible to oxidative damage. This finding was in accordance with the previous study showing that hypertension induced by blocking NO synthesis is associated with increase oxidative stress (Duarte et al., 2002). In addition, a previous study has proposed that oxidative stress contributes to the generation or maintenance of hypertension via inactivation of NO (Ortiz et al., 2001). MCHF treatment decreased the level of lipid peroxidation in L-NAME treated rat. The antioxidant of MCHF may prevent the free radicle produced by L-NAME. This result confirmed that MCHF has antioxidant property, which might be partially responsible for alleviation of hypertension in NO deficiency rats.

Thus, the increased NO production and decreased oxidative load after MCHF treatment may lead to blood pressure reduction. MCHF was found to be herbal formulation with potential ability to treat hypertension.

Toxicity information of herbal formulation by scientific approaches makes people have confidence in the safety of herbal uses. In this study, the hepatotoxicity of MCHF was evaluated by using HepG2 cells as a model. After MCHF treatment for 24 hours, the formulation showed cytotoxic effects at the concentration of 5 mg/mL. When cells were continuously exposed to the MCHF formulation for 7 days, the formulation at 0.05 mg/mL significantly caused a toxic effect on HepG2 cells. These results are consistent with those of other studies demonstrating that MCHF at the concentration higher than that used in humans (1000 mg/kg) significantly raised the ALT levels after treatment in rats for 4 weeks (Khumkhrong & Kunkaew, 2012). These results suggested the possibility of hepatotoxicity in the high dose formulation. However, this finding is in agreement with the study reporting that MCHF was not found to have cytotoxic effect on human white blood cells (Madeachumporn N, 2014). Thus, further studies on toxicity testing using normal human liver cells should be performed in order to confirm the toxicity and safety profile of this formulation.

6. Conclusion

The present study demonstrated that MCHF is able to reduce blood pressure. This effect is likely to be caused by restoring NO levels and suppression of oxidative stress by MCHF. The hepatotoxicity may be increased when high doses of MCHF are taken. However, further studies are needed to explore the mechanism of antihypertensive effect of MCHF in detail.

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