Title  Polyphenols from Lonicera caerulea L. berry attenuate experimental nonalcoholic steatohepatitis by inhibiting proinflammatory cytokines productions and lipid peroxidation

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Received: 29/09/2016; Revised: 17/11/2016; Accepted: 24/11/2016

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201600858.

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Abbreviations: α-SMA, alpha-smooth muscle actin; FoxO1, forkhead box protein O1; G-CSF, granulocyte colony-stimulating factor; GGT, gamma-glutamyl transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; GOT, glutamic oxaloacetic transaminases; GPT, glutamate-pyruvate transaminases; HFD, high fat diet; HO-1, heme oxygenase-1; KC, keratinocyte-derived cytokine; LCP, Lonicera caerulea L. polyphenols; MCP-1, monocytes chemotactic protein-1; MIP, macrophage inflammatory protein; MnSOD, manganese-dependent superoxide dismutase; NASH, Nonalcoholic steatohepatitis; ND, Normal diet; Nrf2, nuclear factor (erythroid-derived 2)-like 2; TBARS, thiobarbituric acid reactive substances; T-cho, total cholesterol, TG, total triacylglycerol; TNF-α, tumor necrosis factor-alpha

Abstract:

Scope: Nonalcoholic steatohepatitis (NASH) is a common disease that closely associated with inflammation and oxidative stress, and Lonicera caerulea L. polyphenols (LCP) are reported to possess both antioxidant and anti-inflammatory properties. This study aimed to study the protective effects and mechanisms of LCP on NASH in a high fat diet (HFD) plus carbon tetrachloride (CCL₄)-induced mouse model.

Methods and results: Mice were fed with HFD containing LCP (0.5-1%) or not, and then administrated with CCL₄ to induce NASH. Liver sections were stained by hematoxylin-eosin stain,
serum transaminases and lipids were measured by clinical analyzer, insulin was examined by ELISA, cytokines were determined by multiplex technology, and hepatic proteins were detected by western blotting. LCP improved histopathological features of NASH with lower levels of lipid peroxidation and cytokines including G-CSF, IL-3, IL-4, MIP-1β, IL-6, IL-5, KC, TNF-α, IL-2, IL-1β, MCP-1, IL-13, IFN-γ, IL-10, IL-12(p70), IL-1α, eotaxin, GM-CSF, MIP-1α, IL-17, and RANTES. Further molecular analysis revealed that LCP increased the expression of Nrf2 and MnSOD, but decreased FoxO1 and HO-1 in the liver of NASH mice.

**Conclusion:** Dietary supplementation of LCP ameliorated inflammation and lipid peroxidation by up-regulating Nrf2 and MnSOD, and down-regulating FoxO1 and HO-1 in NASH.
Dietary supplementation of LCP, the polyphenols from *Lonicera caerulea* L. berry, ameliorated inflammation, lipid peroxidation, and insulin resistance in the experimental NASH model by suppressing proinflammatory cytokines productions, up-regulating the expression of Nrf2 and MnSOD, and down-regulating the expression of FoxO1 and HO-1.

**Keyword:**
**Lonicera caerulea** L.; polyphenols; nonalcoholic steatohepatitis; cytokines; lipid peroxidation; Nrf2; HO-1

1. Introduction

Nonalcoholic steatohepatitis (NASH) is a common liver disease in the modern society, and the pathogenesis is unclear yet. Although obesity is correlated with an increased risk of NASH [1], it is not the decisive factor since NASH can be developed in lean people [2]. Recent studies suggested that inflammatory and metabolic disorders caused by the dysbiosis of gut microbiota potentially accelerate the progression of NASH [3, 4], and proinflammatory cytokines and oxidative stress play an important role in promoting hepatic inflammation, lipid peroxidation, and fibrosis [5, 6].

Natural phytochemicals such as polyphenols contribute to the attenuation of both oxidative stress and inflammation. *Lonicera caerulea* L. berry, commonly called as blue honeysuckle or haskap, is native to Siberia, China and Japan, and recently accepted as a healthy berry in Western countries such as United States [7], Canada [8], and Czech Republic [9]. *Lonicera caerulea* L. berry has been reported to possess anti-microbial [10], antioxidant [11, 12], and anti-inflammatory activities [8, 9, 13], and the major bioactive components have been identified as cyanidin 3-glucoside and (-)-epicatechin [13].

Based on the above information, this study aimed to investigate the protective effects and mechanisms of *Lonicera caerulea* L. polyphenols (LCP) on NASH. As NASH is a long-term disease that with few or no symptoms in the early stages, and previous study revealed that mice fed with high fat diet (HFD) exhibit typical features of NASH after more than 60 weeks [14], the progressive NASH models are commonly used in laboratory studies. The “two-hit” hypothesis [15], in which first hit causes fat accumulation in hepatocytes, and second hit induces inflammation and fibrosis, is widely accepted for building NASH model. Carbon tetrachloride (CCL₄) is a well-known hepatotoxicant used in experimental models to induce hepatic inflammation and fibrosis [16], and a recently study
indicated that HFD with multiple administration of CCL$_4$ successfully induced pathological features of NASH in a mouse model [17]. Thus, we attempted to use a HFD plus CCL$_4$-induced mouse model to explore the protective effects of LCP on NASH, and further clarify the mechanisms by investigating the expressions of both cytokines and oxidative stress response proteins.

2. Materials and Methods

2.1 Chemicals and Reagents

CCL$_4$ (≥99.5%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lard oil was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Antibodies against alpha-smooth muscle actin (α-SMA), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), manganese-dependent superoxide dismutase (MnSOD), heme oxygenase-1 (HO-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against forkhead box protein O1 (FoxO1) and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). LCP was extracted as described previously [13], briefly, Lonicera caerulea L. berries harvested in Jilin region of China were homogenized in 75% aqueous ethanol (250 g/L) for 60 min, and then filtered under reduced pressure. The filtrates were purified on a column packed with nonionic polystyrene-divinylbenzene resin (D101, Shanghai, China), and then freeze-dried into powder. The obtained phenolic fraction was analyzed by HPLC at 280 and 520 nm. According to the retention profiles of standard samples, C3G (59.5%) and EC (25.5%) were identified as the major phenolic components at 280 nm while other minor anthocyanins including peonidin 3-glucoside (7.2%), pelargonidin 3-glucoside (2.3%), peonidin 3-rutinoside (1.9%), cyanidin 3-rutinoside (1.8%), and cyanidin 3,5-diglucoside (1.3%) were also detected at 520 nm. The quantitative analysis indicated that each milligram of LCP contains 0.37 mg C3G and 0.23 mg EC.

2.2 Mouse NASH model

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The animal experimental protocol was drafted according to the guidelines of the Animal Care and Use Committee of Kagoshima University (Permission No. A12005). Male C57BL/6N mice (5 weeks of age) from Japan SLC Inc. (Shizuoka, Japan) were housed separately in cages with wood shavings bedding under controlled light (12 h light/day) and temperature (25°C), and free access to water and feed. After acclimatization for one week (Day 0), the mice were randomly divided into six groups (n=4): Normal diet (ND), ND+LCP1%, HFD, HFDCCl4, HFDCCl4+LCP0.5%, and HFDCCl4+LCP1%, and fed with the corresponding diets as described in Supplemental Table 1. After 30 days (Day 30), the mice in HFDCCl4, HFDCCl4+LCP0.5%, and HFDCCl4+LCP1% groups were intraperitoneally injected (i.p.) with CCL4 at a dose of 0.05 ml/kg body weight (BW) every 3 days to induce NASH until Day 45. The dosage of CCL4 used in this study was based on our pilot test. Mice were sacrificed at Day 45 after overnight fasting.

2.3 Hepatic histology

Mice liver were collected at day 45 and sectioned by using a freezing microtome system (Yamato, Saitama, Japan) according to the manufacturer’s manual. Obtained liver sections were then stained with hematoxylin-eosin (H&E) staining, and observed under a fluorescence microscope (Keyence, Tokyo, Japan).

2.4 Hepatic protein extraction and western blot assay

The total proteins of liver were obtained by using RIPA buffer [13]. Briefly, equal amounts of liver tissues were homogenized in RIPA buffer (0.1 g/ml) with a Speed-Mill PLUS homogenizer (Analytik Jena, Jena, Germany). The supernatant proteins were obtained by being centrifuged at 13,500 g for 5 minutes at 4 °C, and the protein concentrations were determined by using a due-binding protein assay kit (Bio-Rad Hercules, CA, USA) according to the manufacturer’s manual. The protein extracts were boiled in SDS sample buffer for 5 min, and equal amounts of protein (40μg) were run on a 10% SDS-PAGE gel before electrophoretically transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membrane was then incubated with specific primary antibody and HRP-conjugated
secondary antibody, following by detection with a LumiVision PRO system (TAITEC Co., Saitama, Japan).

2.5 Measurement of SOD activity and TBARS level in liver

The activity of superoxide dismutase (SOD) and the level of thiobarbituric acid reactive substances (TBARS) in the liver were measured with their respective assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer’s manual.

2.6 Measurement of transaminases, lipids, glucose, and insulin in serum

Blood sera were obtained by centrifuging at 1500 g for 10 min after coagulation at day 45. The levels of glutamic oxaloacetic transaminase (GOT), glutamate-pyruvate transaminase (GPT), and gamma-glutamyl transferase (GGT), total cholesterol (T-cho), HDL cholesterol (HDL-c), total triacylglycerol (TG), and glucose were measured with an automated analyzer for clinical chemistry (Arkray, Kyoto, Japan). The insulin concentration was measured with an ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), according to the manufacturer’s manual.

2.7 Determination of cytokines by multiplex technology in serum

Serum levels of cytokines including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, keratinocyte-derived cytokine (KC), monocytes chemotactic protein 1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES, and tumor necrosis factor-alpha (TNF-α) were measured with a Bio-Plex Pro Mouse Cytokine 23-Plex Panel kit (Bio-Rad Hercules, CA, USA) by using a Bio-Plex 200 System according to the manufacturer’s manual.

2.8 Statistical analysis
Results were expressed as mean ± SD. The significant differences between groups were analyzed by one way ANOVA tests, followed by Fisher’s LSD and Duncan’s multiple range tests with the SPSS statistical program (version 19.0, IBM Corp., Armonk, NY, USA). *p < 0.05 and **p < 0.01.

3. Results

3.1 LCP improved histopathological features of NASH

To evaluate the fat deposition in mice, the intra-abdominal fat/BW and liver/BW ratios were first measured. As shown in Fig. 1A&B, HFD caused significant increase in the intra-abdominal fat/BW ratio but not liver/BW ratio, while HFD plus CCL₄ caused significant increase in both, as CCL₄ caused a decline in intra-abdominal fat and BW (Supplemental Fig. 1), but increased liver weight. Liver section then showed that HFD caused accumulation of lipid droplets and inflammatory cells (arrow) in liver, while HFD plus CCL₄ caused not only accumulation of more lipid droplets and inflammatory cells, but also severe necrosis (arrow) (Fig. 1C). Supplement with 0.5-1% of LCP in the diet reduced intra-abdominal fat/BW (p < 0.01) and liver/BW (p < 0.05) ratios, and markedly ameliorated hepatic fat accumulation, inflammatory cell infiltration, and necrosis. To investigate the degree of fibrosis, the expression of α-SMA, an indicator of fibrosis, was further detected in mice liver. Fig. 1D showed that the expression of α-SMA was raised to 1.5 (p < 0.01 vs ND) and 3.1 (p < 0.01 vs ND) folds in HFD and HFDCCl₄ group, but decreased to 1.7 (p < 0.01 vs HFDCCl₄) and 1.4 (p < 0.01 vs HFDCCl₄) folds by supplementing with 0.5% and 1% of LCP, respectively. As a sample control, 1% of LCP showed no significant influence on the normal mice.

3.2 LCP decreased serum transaminases and lipids

Serum transaminases and lipids are useful indicators of liver damage. As shown in Fig. 2, serum levels of GOT (A), GPT (B), and GGT (C) were increased (p < 0.01) in HFDCCl₄ group but not
HFD group, and dose-dependently reduced by supplementing with 0.5-1% of LCP. Analysis on serum lipids then revealed that the level of TG (Fig. 2D) was increased \((p < 0.01)\) in both HFD and HFDCL\(_4\) group, while the T-cho/HDL-c ratio (Supplemental Fig. 2A), an index of insulin resistance syndrome [18], was increased \((p < 0.05)\) in HFDCL\(_4\) group only. Supplement with LCP decreased both the TG level \((p < 0.01)\) and T-cho/HDL-c ratio \((p < 0.05)\). Further analysis on serum levels of insulin and glucose revealed that the serum insulin/glucose ratio, an indicator of insulin sensitivity [19], was significantly increased \((p < 0.01)\) in both HFD and HFDCL\(_4\) group, and decreased \((p < 0.01)\) by LCP (Supplemental Fig. 2B).

### 3.3 LCP decreased serum levels of multiple cytokines

As cytokines play a significant role in inflammation, twenty-three kinds of cytokines were determined in serum to understand the modulation of cytokine network by LCP. As shown in Fig.3, serum levels of all the analyzed cytokines except IL-9 and IL-12(p40) were increased in HFDCL\(_4\) group, while only KC, TNF-\(\alpha\), and IL-2 \((p < 0.05)\) were increased in HFD group. Especially, the levels of G-CSF, IL-3, IL-4, MIP-1\(\beta\), IL-6, IL-5, KC, TNF-\(\alpha\), IL-2, IL-1\(\beta\), MCP-1, IL-13 and IFN-\(\gamma\) were raised to over two folds in HFDCL\(_4\) group. Supplement with 0.5-1% of LCP in diet decreased the serum levels of G-CSF, IL-3, IL-4, MIP-1\(\beta\), IL-6, IL-5, KC, TNF-\(\alpha\), IL-2, IL-1\(\beta\), MCP-1, IL-13, IFN-\(\gamma\), IL-10, IL-12(p70), IL-1\(\alpha\), eotaxin, GM-CSF and MIP-1\(\alpha\) \((p < 0.01)\), as well as IL-17 and RANTES \((p < 0.05)\), but showed no significant influence on IL-9 and IL-12(p40) (data not shown).

### 3.4 LCP improved hepatic antioxidant capacity

Oxidative stress is another critical factor in the pathogenesis of NASH [6]. To evaluate the oxidative status in liver, the activity of SOD and the level of TBARS were measured. As shown in Fig. 4A, SOD activity was decreased \((p < 0.01)\) in HFDCL\(_4\) group, but recovered by supplementing with 1% LCP.
of LCP. On the contrary, the level of TBARS, the product of lipid peroxidation, was increased in both HFD \((p < 0.05)\) and HFDCCL\(_4\) \((p < 0.01)\) group, and dose-dependently decreased by 0.5-1\% of LCP (Fig. 4B). Further determination of the oxidative stress response proteins in liver showed that the expression of Nrf2, a key transcription factor that regulates the expression of antioxidant proteins, was decreased to 0.6 \((p < 0.01 \text{ vs ND})\) and 0.5 \((p < 0.01 \text{ vs ND})\) folds in HFD and HFDCCL\(_4\) group, but recovered to 0.7 \((p < 0.01 \text{ vs HFDCCL}\_4)\) and 0.9 \((p < 0.01 \text{ vs HFDCCL}\_4)\) folds in the group treated with 0.5\% and 1\% of LCP, respectively (Fig. 4C, 1\(^{st}\) band). Potential downstream targets including MnSOD and HO-1 were then detected, and the expression of MnSOD (Fig. 4C, 2\(^{nd}\) band) showed similar trend as Nrf2. On the other hand, the expression of HO-1 was reduced to 0.6 \((p < 0.01 \text{ vs ND})\) folds in HFD group, but increased to 1.8 \((p < 0.01 \text{ vs ND})\) folds in HFDCCL\(_4\) group (Fig. 4C, 3\(^{rd}\) band). Thus, we investigated the expression of FoxO1, another transcription factor that regulates HO-1 [20]. The results revealed that the level of FoxO1 was also increased to 1.8 \((p < 0.01 \text{ vs ND})\) folds in HFDCCL\(_4\) group (Fig. 4C, 4\(^{th}\) band). Supplement with 0.5-1\% of LCP recovered the expression of Nrf2 and MnSOD, but decreased the expression of HO-1 and FoxO1 significantly \((p < 0.01)\).

### 4. Discussion

An appropriate model is important in order to study the pathogenesis and prevention of NASH. In this study, HFD-fed mice became obese with fatty liver but no steatohepatitis, while the mice received two-hit by HFD plus CCL\(_4\) administration showed histopathological features of NASH including fatty liver, hepatocyte injury, inflammation, and fibrosis.

In the progression of NASH, cytokines not only provoke the local inflammatory response in the liver, but also induce inflammatory cells infiltration to promote fibrosis. In this study, HFD plus CCL\(_4\) increased the production of twenty-one kinds of cytokines, and the levels of G-CSF, IL-3, IL-4, MIP-1\(\beta\), IL-6, IL-5, KC, TNF-\(\alpha\), IL-2, IL-1\(\beta\), MCP-1, IL-13 and IFN-\(\gamma\) were increased to over two
folds. Among the cytokines, G-CSF and IL-3 promote proliferation and differentiation of hematopoietic stem cells in response to liver injury [21, 22], and IL-4, IL-5, and IL-13 are considered as the anti-inflammatory cytokines against excessive inflammation and self-immunity [23]. IL-6, TNF-α, IL-2, IL-1β, and IFN-γ might be the potential primary proinflammatory cytokines in the pathogenesis of NASH, as chemokines including MIP-1β, KC, and MCP-1 were potentially mediated by IL-1β [24], TNF-α [25], and IL-6 [26], respectively. Dietary supplementation of LCP decreased both pro- and anti-inflammatory cytokines, which suggesting that LCP ameliorated NASH by decreasing the production of proinflammatory cytokines, rather than promoting the production of anti-inflammatory cytokines.

Oxidative stress is considered as another important factor in the pathogenesis of NASH [6]. In this study, HFD and CCL₄ decreased the expression of antioxidant proteins (SOD, Nrf2, and MnSOD) and enhanced lipid peroxidation (TBARS) in the liver. Interestingly, HO-1, one of the downstream antioxidant defense proteins of Nrf2, was decreased in HFD-fed mice but increased in the mice administrated with HFD plus CCL₄. Although HO-1 has been reported to contribute to ameliorate obesity and fatty liver [27], a recent study suggested that HO-1 actually drives metaflammation and insulin resistance in metabolic disease [28]. FoxO1 is proved as another regulator of HO-1 [20], and it is activated in insulin receptor substrate-knockout mice [29]. The overexpression of FoxO1 and HO-1 can further aggravate insulin resistance and promote the production of proinflammatory cytokines [28, 30]. Our data in this study revealed that FoxO1 was increased markedly in the mice administrated with HFD plus CCL₄ but not HFD only. These results suggested that HO-1 was possibly reduced due to the low level of Nrf2 in the early stage of obesity, but increased with the overexpression of FoxO1 induced by metabolic dysfunction in NASH. LCP increased the expression of Nrf2 and MnSOD, but decreased the expression of FoxO1 and HO-1, and further ameliorated the lipid peroxidation and insulin resistance in NASH mice.

In conclusion, dietary supplementation of LCP ameliorated inflammation, lipid peroxidation, and insulin resistance in the experimental NASH model by suppressing proinflammatory cytokines.
production, up-regulating the expression of Nrf2 and MnSOD, and down-regulating the expression of FoxO1 and HO-1.

Mr. Shusong Wu is the primary investigator in this study. Mr. Satoshi Yano and Dr. Ayami Hisanaga participated in the animal experiments. Dr. Xi He and Dr. Jianhua He participated in the sample extraction and purification. Dr. Kozue Sakao participated in sample analysis. Dr. De-Xing Hou designed this study and wrote the manuscript as corresponding author.

This work was partially supported by grant-in-aid for scientific research of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (18580125) and Core Research Program 1515 of Hunan Agricultural University. Mr. Shusong Wu received a studentship supported by China Scholarship Council (CSC).

The authors have declared no conflict of interest.

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Legends to Figures

**Figure 1**  LCP improved histopathological features of NASH. (A) The weight percentage of fat to BW. (B) The weight percentage of liver to BW. (C) Representative liver sections from each group (H&E stain, original magnification ×200). (D) The representative blot of α-SMA protein in liver by western blotting. The induction fold of α-SMA was calculated as the intensity of the treatment relative to that of control normalized to GAPDH by densitometry. The data represent mean ± SD of four mice. *p < 0.05 and **p < 0.01.
Figure 2  LCP decreased serum transaminases and lipids. (A) Serum GOT level. (B) Serum GPT level. (C) Serum GGT level. (D) Serum TG level. The data represent mean ± SD of four mice. *p < 0.05 and **p < 0.01.
Figure 3  LCP decreased serum levels of multiple cytokines. The levels of 23 kinds of cytokines were measured by multiplex technology, and arranged in an order from high to low change in the experimental NASH model. The data represent mean ± SD of four mice. *p < 0.05 and **p < 0.01.
Figure 4 LCP improved hepatic antioxidant capacity. (A) SOD activity in liver. (B) The level of TBARS in liver. (C) The representative blots of Nrf2, MnSOD, HO-1, and FoxO1 protein in liver by western blotting. The induction folds of the proteins were calculated as the intensity of the treatment relative to that of control normalized to GAPDH by densitometry. The data represent mean ± SD of four mice. *p < 0.05 and **p < 0.01.
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