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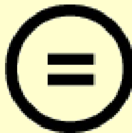

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의학석사 학위논문

Eupatilin attenuates liver injury induced by methionine choline-deficient diet in mice

Eupatilin이 methionine choline 결핍 식이에 의한 간 손상에 미치는 영향

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서울대학교 대학원
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이 동 현

A thesis of the Master’ s degree

Eupatilin attenuates liver injury induced by methionine choline-deficient diet in mice

February 2014

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Eupatilin이 methionine choline 결핍 식이에 의한 간 손상에 미치는 영향

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Eupatilin attenuates liver injury induced by methionine choline-deficient diet in mice

by

Dong Hyeon Lee, M.D.

A Thesis Submitted to the Department of Internal Medicine
in Partial Fulfillment of the Requirements
for the Degree of Master of Philosophy in Medicine
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Approved by thesis committee:

Professor ________________ Chairman

Professor ________________ Vice Chariman

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논문 제목: Eupatilin 인 methionine choline 결핍 식이에 의한 간손상에 미치는 영향

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// Abstract

Eupatilin attenuates liver injury induced by methionine choline-deficient diet in mice

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Introduction: The prevailing hypothesis in pathogenesis of nonalcoholic steatohepatitis (NASH) consists of two steps: excessive fat accumulation and hepatocyte injury by oxidative stress, abnormal cytokines, mitochondrial dysfunction, and/or endoplasmic reticulum (ER) stress. Eupatilin, a pharmacologically active ingredient found in Artemisia asiatica, has been established anti-oxidative, anti-inflammatory, and cytoprotective agents. In the present study, we evaluated whether eupatilin prevents the development of NASH in mice.

Methods: C57BL/6 mice were fed methionic choline-deficient (MCD) diet with or without eupatilin (50 or 100 mg/kg per body weight) for 8 weeks. The effects of eupatilin on the development and progression of NASH and underlying mechanism were studied.

Results: Eupatilin attenuated the liver injury and contributed to histological improvements, including non-alcoholic fatty liver disease activity score (NAS) through suppression of hepatic inflammation, oxidative stress, and ER stress. However, pharmacological effect of eupatilin was not sufficient to reduce the NAS less than 5, which corresponded to NASH.

Conclusions: Modulation of oxidative and ER stress by eupatilin was not sufficient to eradicate development of NASH. However, eupatilin ameliorated hepatocyte injury and NAS. Further studies are needed to maximize the preventative effects of eupatilin in NASH by dose increase or combination therapy.

Keywords: Nonalcoholic fatty liver disease, Fatty liver, Eupatilin, Lipogenesis, Endoplasmic reticulum stress, Oxidative stress

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List of abbreviations and symbols

Acox1 peroxisomal acyl-coenzyme A oxidase 1

BBC3/PUMA BCL2 binding component 3/ p53 upregulated modulator of apoptosis

Ccl2 chemokine (C-C motif) ligand 2

CHOP CCAAT/enhancer-binding protein homologous protein

Cpt2 carnitine palmitoyltransferase II

DAPI 4’,6-diamidino-2-phenylindole dihydrochloride

p-eIF2α phospho-eukaryotic initiation factor 2α

ER endoplasmic reticulum

FASN or Fasn fatty acid synthase

GSH glutathione

HCC hepatocellular carcinoma

p-JNK phospho-c-Jun N-terminal kinase

MCD methionine-choline deficient

MCS methionine-choline sufficient

NAFLD nonalcoholic fatty liver disease

NAS NAFLD activity score

NASH nonalcoholic steatohepatitis

NR1H3 nuclear receptor subfamily 1, group H, member 3

OA oleic acid

OD optical densities

PA palmitic acid

Sod2 superoxide dismutase 2

SREBF1 or Srebf1 sterol regulatory element-binding transcription factor 1

Tgfb1 transforming growth factor beta 1

Timp1 TIMP metallopeptidase inhibitor 1

Tnf tumor necrosis factor

TUNEL terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

Introduction

Nonalcoholic steatohepatitis (NASH) is the gravest presentation of nonalcoholic fatty liver disease (NAFLD) and one of serious outcomes related with the current obesity epidemic.[1] One-third of NAFLD proceed to NASH, which is potential source of hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC).[2] The leading hypothesis in the pathogenesis of NASH is two-hit theory, proposed by James and Day.[3] This hypothesis consists of intrahepatic lipid accumulation followed by liver injury induced by oxidative stress, abnormal cytokines, mitochondrial dysfunction, and/or endoplasmic reticulum (ER) stress. However, there are still many uncertainties in pathogenesis of NASH. The possible mechanisms as mentioned above may not fully explain the diversity of symptoms and physiologic process of disease progression. At present, a multimodal treatment for each component of metabolic syndrome, including obesity, insulin resistance, hypertension, and dyslipidemia, is the only effective therapeutic option for improving NASH.[4]

Eupatilin (5,7-dihydroxy-3,4,6-trimethoxyflavone), one of the major pharmacologically active ingredients found in *Artemisia* species, has been reported to exert anti-oxidative, anti-inflammatory activity, and cytoprotective effects against experimentally induced gastrointestinal and pancreatic damage.[5-8] It has also been reported to reduce liver injury in animal models.[9] Until yet, however, no trials have been conducted whether eupatilin has

protective effects against NASH. If eupatilin exert anti-oxidative, anti-inflammatory, or cytoprotective activities in hepatocytes, it could be a potential therapeutics for NASH, according to two-hit theory.

In this study, we examined whether eupatilin exerts prophylactic activities against NASH occurrence and underlying mechanisms.

Materials and methods

Materials and reagents

Eupatilin, the ethanol extract of *Artemisia asiatica* at a purity of >99.5% was kindely provided by Dong-A Pharmaceutical.

Cell line and culture

Human HCC cell lines HepG2 and Huh-BAT cells (Huh-7 cells stably transfected with a bile acid transporter[10]) were used in this study. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100,000 U/L penicillin, and 100 mg/L streptomycin. In all experiments, cells were serum starved overnight in order to avoid the effects of serum-induced signaling.

Real-time reverse transcription-PCR

Total RNA was extracted from the cells and cDNA templates were prepared, as described previously in detail.[11] The mRNA expression of *CHOP, BBC3/PUMA, NR1H3, FASN, SREBF1, Ccl2, Tnf, Sod2, Acox1, Cpt2, Timp1*, *Tgfb1, Nr1h3, Fasn, and Srebf1* were quantitated using real-time RT-PCR. The sequences of the forward and reverse primers for genes presented above are shown in Table 1. Relative quantification was calculated using the $2^{-\Delta\Delta C_T}$ method and was normalized based to *GAPDH* expression level.

Organism	Primer	Direction	Sequence
Human	*CHOP*	Forward	5' -AGG CAC TGA GGG TAT CAT GTT-3’
		Reverse	5’- CTG TTT CCG TTT CCT GGT TC-3’
	BBC3/PUMA	Forward	5’- GAC GAC CTC AAC GCA CAG TA -3’
		Reverse	5’- AGG AGT CCC ATG ATG AGA TTG T -3’
	NR1H3	Forward	5'- TGG ACA CCT ACA TGC GTC GCA A -3'
		Reverse	5'- CAA GGA TGT GGC ATG AGC CTG T -3'
	FASN	Forward	5'- TTC TAC GGC TCC ACG CTC TTC C -3'
		Reverse	5'- GAA GAG TCT TCG TCA GCC AGG A -3'
	SREBF1	Forward	5'- ACT TCT GGA GGC ATC GCA AGC A -3'
		Reverse	5'- AGG TTC CAG AGG AGG CTA CAA G -3'
Mouse	*Ccl2*	Forward	5'- CAG GTC CCT GTC ATG CTT CT -3'
		Reverse	5'- TCT GGA CCC ATT CCT TCT TG -3'
	Tnf	Forward	5'- CCA TTC CTG AGT TCT GCA AAG -3'
		Reverse	5'- GCA AAT ATA AAT AGA GGG GGG C -3'
	Sod2	Forward	5'- CTG GAC AAA CCT CAG CCC TAA C -3'
		Reverse	5'- AAC CTG AGC CTT GGA CAC CAA C -3'
	Acox1	Forward	5’- TGG TAT GGT GTC GTA CTT GAA TGA C -3’
		Reverse	5’- AAT TTC TAC CAA TCT GGC TGC AC -3’
	Cpt2	Forward	5’- GCC CAG CTT CCA TCT TTA CT -3’
		Reverse	5’- CAG GAT GTT GTG GTT TAT CCG C -3’
	Timp1	Forward	5’- GCA TCT CTG GCA TCT GGC ATC -3’
		Reverse	5’- GCG GTT CTG GGA CTT GTG GGC -3’
	Tgfβ1	Forward	5'- CAC CAT CCA TGA CAT GAA CC -3'
		Reverse	5'- TGG TTG TAG AGG GCA AGG AC -3'
	Nr1h3	Forward	5'- ATC GCC TTG CTG AAG ACC TCT G -3'
		Reverse	5'- CTG CTT TGG CAA AGT CTT CCC G -3'
	Fasn	Forward	5’- AGC GGC CAT TTC CAT TGC CC -3’
		Reverse	5’- CCA TGC CCA GAG GGT GGT TG -3’
	Srebf1	Forward	5’- AAC GTC ACT TCC AGC TAG AC -3’
		Reverse	5’- CCA CTA AGG TGC CTA CAG AGC -3’

Table 1. The sequences of the forward and reverse primers

Immunoblot analysis

Cells were lysed for 20 min on ice with lysis buffer (50 mM Tris–HCl (pH 7.4); 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 lg/mL aprotinin, leupetin, and pepstatin; 1 mM Na3VO4; and 1 mM NaF) and centrifuged at 14,000g for 10 min at 4 °C. Samples were resolved with 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and blotted using appropriate primary antibodies with peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA, USA). Bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, USA) and exposed to film (X-Omat; Kodak, Hannover, Germany). The primary antibodies used were: anti-phospho-c-Jun N-terminal kinase (JNK), and anti-phospho-eukaryotic initiation factor 2α (eIF2α) purchased from Cell Signaling Technology (Beverly, MA, USA); anti-caspase 7, anti-caspase 8, anti-caspase 9, and anti-caspase 12 from Pharmingen (San Diego, CA, USA); and anti-phospho-eIF2α, anti-BIP, anti-ATF-6, anti-CHOP/GADD153, anti-XBP-1 from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

Oil Red O staining

Cells were stained with Oil Red O to assess lipid content according to the standard protocol.[12] In brief, cells were fixed with 4 % formaldehyde. The fixed cells were stained with freshly prepared Oil Red O solution for 1 h at 37°C. After Oil Red O staining, cells were rinsed in 60 % isopropanol for

30 s, followed by washing with PBS before microscopic examination.

Quantification of apoptosis

The levels of apoptosis were evaluated using the nuclear binding dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) to measure apoptotic cells by fluorescence microscopy (Zeiss, Germany). The cells were treated with DAPI for 30 min and evaluated by fluorescence microscopy. Apoptotic cells were defined as those containing nuclear fragmentation and condensed chromatin. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total counted cells ×100. For each treatment, a minimum of 400 cells was counted.

Experimental animals and diets

The use and care of animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Seoul National University Hospital. Male C57BL/6 mice were purchased from Orient Bio, Inc. Animals were acclimated to laboratory conditions for a week prior to experimentation, and were housed in animal rooms with environmentally controlled temperature (22±2°C), relative humidity (50±10%), and ventilation (12–18 times/h), under a 12 h light/dark cycle. Proper diet for each groups and autoclaved water were provided *ad libitum*. All animal procedures were in accord with the "Guide for the Care and Use of Laboratory Animals" issued by the Institute of Laboratory Animal Resources Commission on Life Sciences, U.S. National Research Council. A total of 20 six-week-old male

C57BL/6 mice were classified into two groups based on diet type. Mice were fed with methionine-choline sufficient (MCS) diet (Dyets, Inc, Bethlehem, PA) (*n*=5), methionine-choline deficient (MCD) diet (Dyets, Inc, Bethlehem, PA) (*n*=15)[13] for 8 weeks. Mice in MCD diet group were divided into three groups: placebo (n=5), 50mg/kg of eupatilin (n=5), and 100mg/kg of eupatilin (n=5). For the 8 weeks of the study, mice were received placebo or eupatilin for five days per a week, intragastrically by oral gavage. After 8 weeks, the animals were weighed and anaesthetized by inhalatory isoflurane so that their blood could be sampled from the retro-orbital plexus for biochemical analysis. The animals were killed at the end of the experiment by inhalatory carbon dioxide.

Histological examination

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin or Sirius red according to standard protocols. In addition, tissue sections were immunostained with rabbit anti-nitrotyrosine (1:300) antibody (Millipore, Billerica, MA) to detect the presence of peroxynitrites using standard procedures.[14] Furthermore, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed with the *in situ* cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Histological activity was assessed using the NAFLD activity score (NAS) which incorporates scores of steatosis, lobular inflammation, and ballooning.[15] The percentage of area labeled by Sirius red or anti-nitrotyrosine

antibody was measured by an image analysis system (Leica OWin, Leica Imaging Systems, Germany) in ten random microscopic fields (×200). Hepatocyte apoptosis was quantified by counting the number of TUNEL-positive cells in ten random microscopic fields (×400).

Statistical analysis

Statistical analysis was performed using SPSS version 19.0 (SPSS, Chicago, Illinois). Data were expressed as the mean±SD. Differences among groups were compared using two-tailed Student’s t-test or one-way ANOVA according to the nature of the data, and Tukey’s honestly significant difference test was employed as a post hoc test to make pair-wise comparisons between treatments in one-way ANOVA. Differences with a P value of <0.05 were considered statistically significant.

Results

Effects of eupatilin on hepatocyte de novo lipogenesis and lipoapoptosis

We first examined the impacts of eupatilin on saturated FFA-induced hepatocyte lipoapoptosis. Lipoapoptosis was induced in HepG2 and Huh-BAT cells by palmitic acid (PA) treatment. As shown in Fig. 1A, eupatilin pretreatment significantly attenuated PA-induced hepatocyte lipoapoptosis. We next tried to identify the PA-induced proapoptotic signals, affected by eupatilin pretreatment. P-EIF2α and p-JNK, markers for activity of endoplasmic reticulum (ER) stress, were induced by PA. The caspase downstream were also activated by PA. However, when cells were pretreated with eupatilin, this signaling was diminished (Fig. 1B). And eupatilin decreased the mRNA levels of *CHOP* and *BBC3/PUMA*, which were ER stress related proapoptotic proteins, as assessed by real-time PCR (Fig. 1C). These observations collectively suggest that the eupatilin inhibited saturated FFA-induced lipoapoptosis by decreasing ER stress. Reduction of intracellular lipid accumulation and activity of ER stress induced caspase pathway was the key mechanisms of protective effect of eupatilin against the PA-induced lipoapoptosis. Oleic acid (OA)-induced intracellular lipid accumulation was reduced in both human hepatocyte cell lines pretreated with eupatilin (Fig. 1D). *De novo* lipogenesis related genes, such as *FASN,* and *SREBF1*, were suppressed in HepG2 and Huh-BAT cells pretreated with eupatilin, too (Fig. 1E).

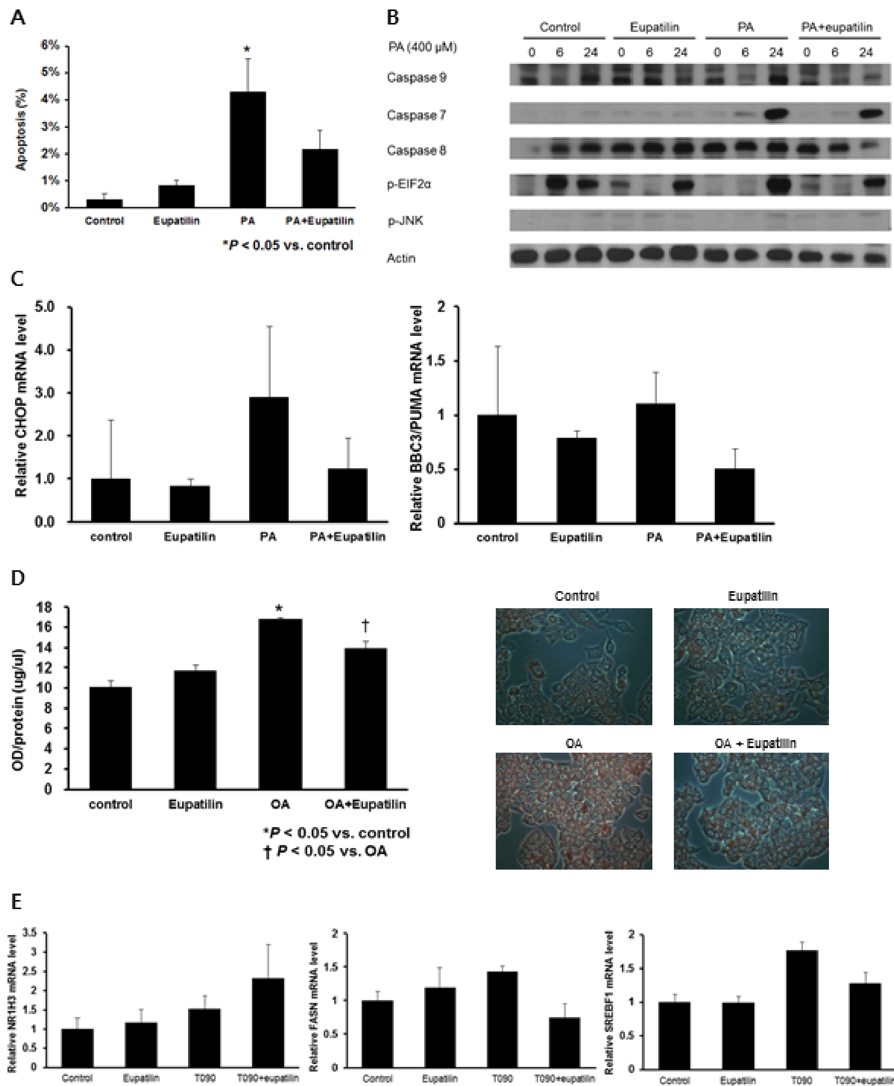

Figure 1. Eupatilin decreased palmitic acid-induced lipoapoptosis and oleic acid-induced lipid accumulation in HepG2 and Huh-BAT cells. **A** Apoptosis was quantified using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays as described in Methods. Eupatilin attenuate palmitic acid (PA)-induced lipoapoptosis. **B** Eupatilin suppressed the PA-induced endoplasmic reticulum (ER) stress and their downstream apoptotic pathway. **C** Eupatilin inhibited the activation of ER stress related proapoptotic protein genes, induced by PA. **D** *De novo* lipogenesis was quantified using measured optical densities (OD) per protein, stained by Oil Red O as described in Methods. *De novo* lipogenesis, induced by oleic acid (OA), was attenuated by eupatilin. **E** Eupatilin decreased the activities of *de novo* lipogenesis related genes.

Effects of eupatilin on MCD diet induced steatohepatitis in vivo

We evaluated whether eupatilin reduced the hepatic injury in MCD diet induced steatohepatitis. As shown in Table 2, MCD diet induced weight loss, ALT elevation, and serum lipid reduction.

	Weight change (%)	Serum ALT (IU/L)	Serum TG (mg/dL)	LDL-cholesterol (mg/dL)
Control diet	13.6 ± 6.8	38.2 ± 3.1	104.4 ± 39.0	4.4 ± 0.5
MCD	-26.0 ± 0.8*	574.5 ± 235.7*	17.2 ± 6.5*	1.8 ± 0.8*
MCD-eupatilin (50 mg/kg)	-26.2 ± 4.2	558.5 ± 500.7	16.5 ± 7.8	2.0 ± 0.8
MCD-eupatilin (100 mg/kg)	-24.6 ± 2.2	490.7 ± 124.5	22.3 ± 8.3	2.1 ± 0.7

* $P < 0.01$ compared to the control diet-fed mice

Table 2. Clinical data of eupatilin pretreatment in MCD diet-induced steatohepatitis mouse model

However, eupatilin did not prevent any of these changes. To quantify hepatic injury in mouse, we used inflammatory foci counting, steatosis grade and NAS.[15] Mean numbers of hepatic inflammatory foci were significantly higher in MCD treated mice (0.00 versus 2.13 per low-power field in the normal diet and MCD diet, respectively; $p < 0.001$; Fig. 2A and B). However, hepatic inflammatory foci were significantly reduced in MCD diet mice treated with eupatilin in a dose-dependent manner (2.13 versus 1.50 versus 1.37 for mice treated with eupatilin at 0, 50, and 100 mg/kg, respectively; p=0.013; Fig. 2A and B). And eupatilin attenuated the level of *Ccl2*, and *Tnf* mRNA, which were cytokines involved in systemic inflammation (Fig. 2C). Steatosis grade (0.00 versus 3.00 in the normal diet and MCD diet, respectively; $p < 0.001$; Fig. 2D) and NAS (0.00 versus 6.80 in the normal diet and MCD diet, respectively; $p < 0.001$; Fig. 2E) were significantly higher in MCD diet mice, too. However, eupatilin only reduced NAS in MCD treated mice (6.80 versus 5.20 versus 5.33 for mice treated with eupatilin at 0, 50, and 100 mg/kg, respectively; p=0.006; Fig. 2E), contrary to steatosis grade (3.00 versus 2.80 versus 2.56 for mice treated with eupatilin at 0, 50, and 100 mg/kg, respectively; p=0.205; Fig. 2D). Furthermore, NAS in mice treated with eupatilin were still over 5, which corresponded to NASH. These observations suggest that the eupatilin inhibited the hepatic inflammation and contributed to histological improvements, however, it had limited rolls in prevention of steatosis and blockage of clinical manifestation of NASH.

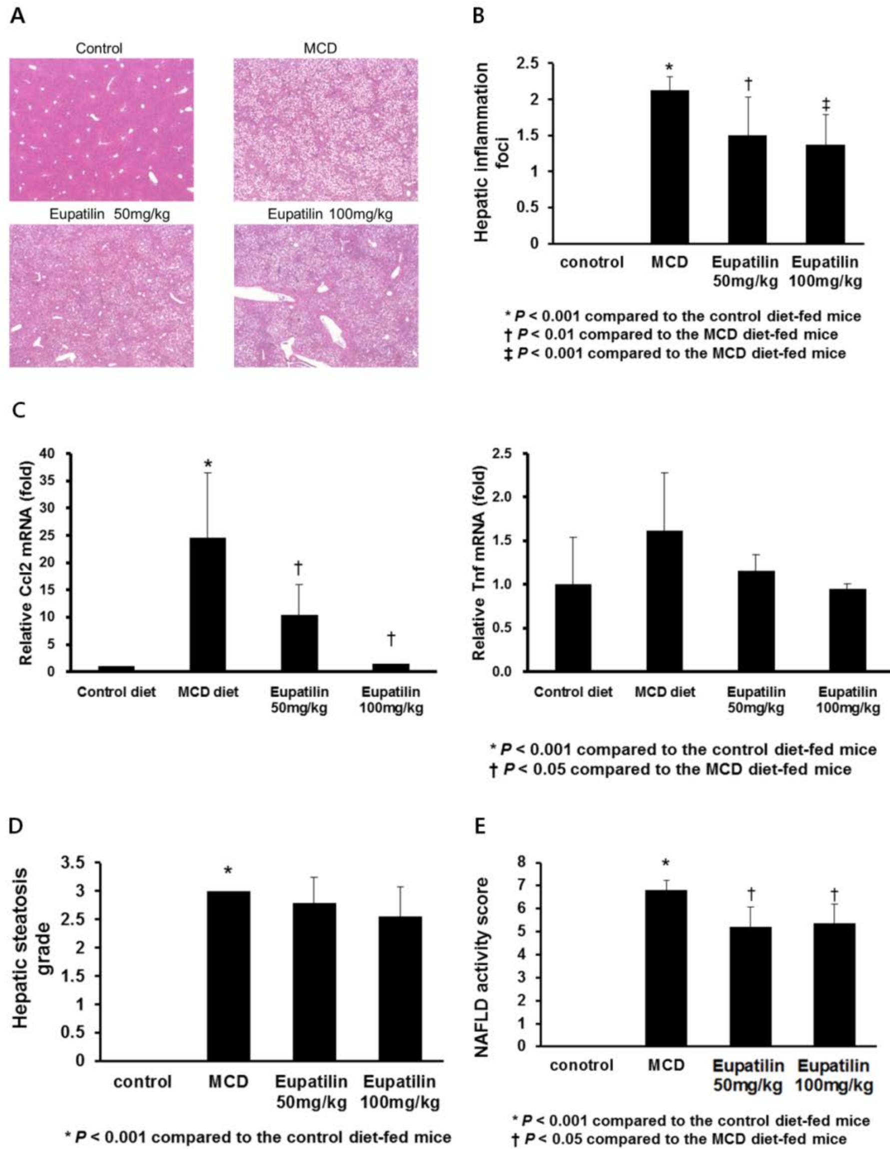

Figure 2. Eupatilin attenuate methionine-choline deficient (MCD) diet-induced steatohepatitis in mouse model. **A, B** Severity of steatosis was quantified using count of inflammatory foci per low power field (x100 magnification). Eupatilin decreased the inflammatory foci count. **C** Eupatilin suppressed levels of systemic inflammatory cytokine genes, induced by MCD diet. **D** Severity of steatosis was quantified using nonalcoholic fatty liver disease (NAFLD) activity score (NAS). NAS declined by eupatilin.

Mechanisms of protective effects of eupatilin on MCD diet induced steatohepatitis

To identify the mechanisms of eupatilin in attenuating liver injury, we next evaluated the anti-oxidative effects of eupatilin. We assessed the percentages of nitrotyrosine stained area in liver tissues. The stained area was more extensive in MCD treated mice (0.67% versus 4.72% in the normal diet and MCD diet, respectively; $p<0.001$; Fig. 3A and B). And the stained areas diminished by eupatilin (4.72% versus 3.22% versus 2.58% for mice treated with eupatilin at 0, 50, and 100 mg/kg, respectively; $p=0.006$; Fig. 3A and B). Eupatilin also attenuated MCD diet induced up-regulation of *Sod2* expression, whereas restored the decreased expression of β-oxidant gene *Acox1* (Fig. 3C and D). These results proposed that eupatilin inhibited liver injury, to act as an anti-oxidative agent *in vivo*.

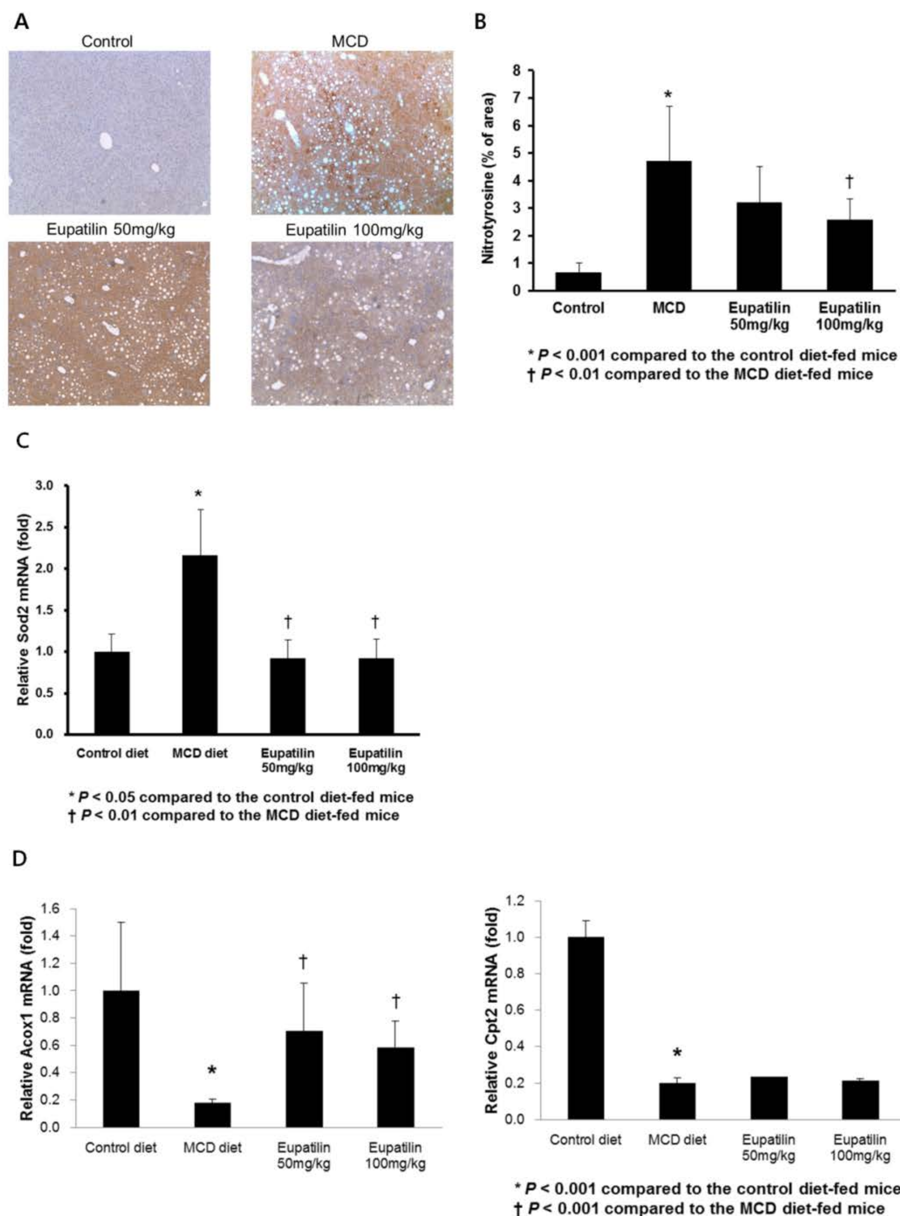

Figure 3. Oxidative stress diminished by eupatilin pretreatment. **A, B** Exposed to oxidative stress was quantified using nitrotyrosine-stained area as described in Methods. Eupatilin attenuated the oxidative stress induced by MCD diet. **C** Eupatilin decreased expression of anti-oxidant gene, **D** whereas eupatilin enhanced the expression of β-oxidant genes.

Moreover, signaling of ER stress and apoptosis, such as p-EIF2α, p-JNK, XBP1, Bip, ATF6, CHOP, and Caspase 12, were all attenuated in mice treated with eupatilin (Fig. 4A). To quantify apoptosis in liver, we used TUNEL staining. The number of TUNEL-positive cells was significantly higher in mice with MCD diet (0.53 versus 10.80 in the normal diet and MCD diet, respectively; $p<0.001$; Fig. 4B and C). And this significantly declined by eupatilin (10.80 versus 7.00 versus 5.60 for mice treated with eupatilin at 0, 50, and 100 mg/kg, respectively; $p<0.001$; Fig. 4B and C). These findings collectively suggest that eupatilin ameliorated MCD diet-induced ER stress and oxidative injury, leading to attenuation of hepatocyte apoptosis.

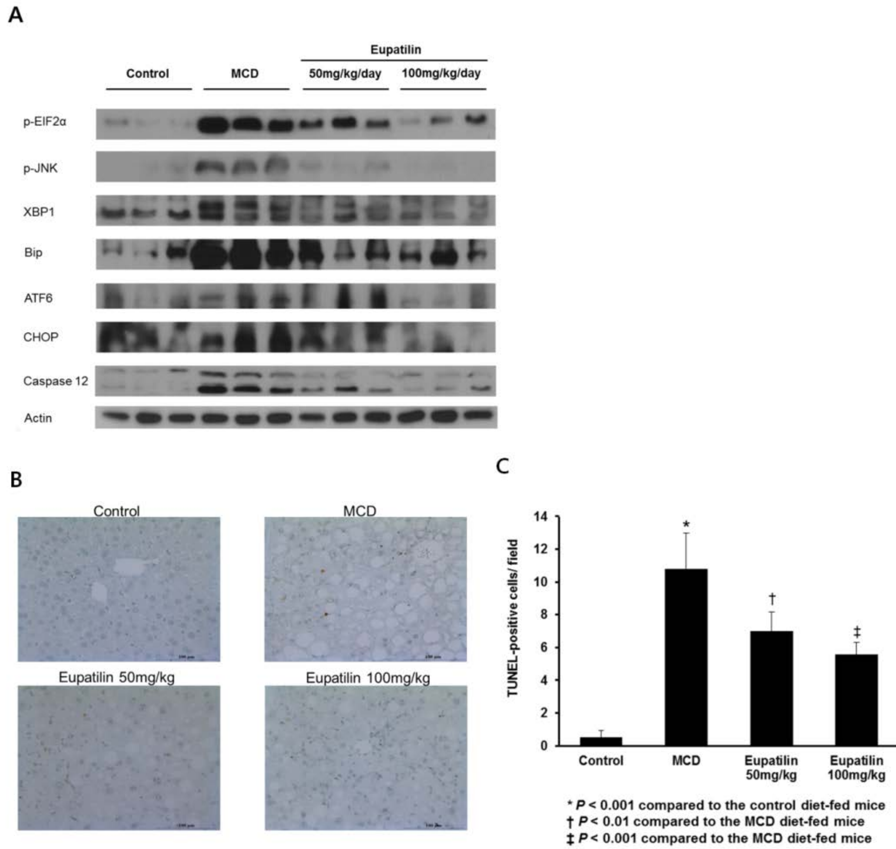

Figure 4. A Eupatilin attenuated the MCD diet-induced ER stress and downstream apoptotic pathway. **B, C** Apoptosis was quantified using TUNEL assays as described in Methods. Eupatilin suppressed MCD diet-induced lipoapoptosis.

Effects of eupatilin on MCD diet induced fibrosis in vivo

The effect of eupatilin on liver fibrosis was studied, simultaneously. The extent of hepatic fibrosis was assessed by Sirius-red staining. The stained areas were more extensive in MCD treated mice (0.45 versus 1.69 in the normal diet and MCD diet, respectively; $p<0.001$; Fig. 5A and B). The areas were attenuated by eupatilin pre-treatment, however, it was not significant (1.69 versus 1.33 versus 1.23 for mice treated with eupatilin at 0, 50, and 100 mg/kg, respectively; $p=0.052$; Fig. 5A and B). Elevated α-SMA, one of myofibroblast formation markers, by MCD diet was suppressed by eupatilin (Fig. 5C). And eupatilin decreased the level of *Timp1*, and *Tgfβ1* mRNA, markers of fibrosis activity (Fig. 5D). These data demonstrate that the eupatilin might have protective effect against hepatic fibrosis, however, it was not sufficient.

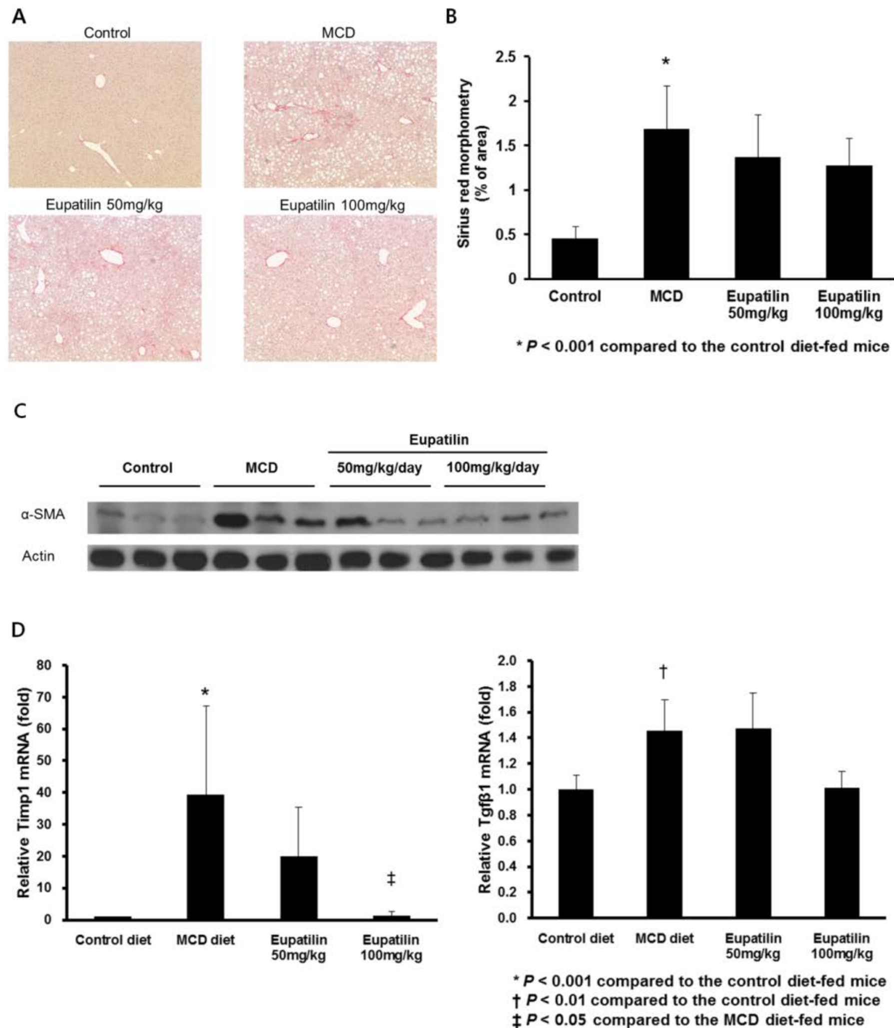

Figure 5. Eupatilin had no effects on MCD diet-induced hepatic fibrosis. **A, B** Hepatic fibrosis was quantified using Sirius-red stained area as described in Methods. Eupatilin was not associated with degree of hepatic fibrosis. **C** Eupatilin inhibited formation of myofibroblast. D And eupatilin decreased the fibrotic activities.

Discussion

Previous studies assessing the effects of eupatilin on NASH liver pathology are lacked. In dietary steatohepatitis model with MCD diet, *Ryu BK et al* reported that eupatilin was effective at attenuating liver injury caused by acetaminophen or CCl_4 via prevention of lipid peroxidation and preservation of hepatic glutathione (GSH).[9] Although eupatilin attenuates oxidative stress in toxic hepatitis, whether eupatilin ameliorates oxidative stress in steatohepatitis remains unclear. And the additional effects of eupatilin in steatohepatitis remain obscure. We demonstrated that eupatilin decreased the hepatic injury induced by MCD diet. Eupatilin decreased hepatocyte damage through suppression of inflammation, oxidative stress, and ER stress. Because hepatic injury was thought to be one of essential process on development of NASH, eupatilin may lead to prevention of development of NASH. In this study, however, pharmacological effect of eupatilin is not enough to eradicate occurrence of NASH.

In the present *in vitro* study, eupatilin attenuates the PA-induced lipoapoptosis and *de novo* lipogenesis. Eupatilin suppressed the activation of p-EIF2α and p-JNK, which were induced by ER stress and upstream of apoptotic pathway. It has recently been supposed that not only hepatocyte apoptosis but also ER stress itself may be driving forces of development of NASH. Considering hypothesis of pathogenesis of NASH, these *in vitro* findings were suggested that eupatilin might be therapeutically useful for prevention of NASH.

However, eupatilin treatment did not suppressed lipogenesis in animal models of steatosis. Biochemical and histologic findings were not shown any association with eupatilin pretreatment. NAS was not changed by eupatilin and expression level of lipogenesis related genes had not enough information to interpret the effect of eupatilin. Eupailin might have limited role in prevention of steatosis, considering as the first step of development of NASH. In contrast, inflammatory reaction and ER stress induced caspase pathway was still suppressed in mice with MCD diet. Eupatilin attenuated transcription of inflammatory cytokines and apoptotic signals. Oxidative stress was diminished by eupatilin pretreatment, too. Eupatilin increased activity of β-oxidation, whereas decreased expression of anti-oxidative genes and nitrotyrosine stained area. These results were demonstrated that eupatilin acts as a protective agent against liver injury and might be still potential preventative agent for NASH. However, pharmacological role of eupatilin was not enough to prevent hepatic fibrosis and development of NASH in this study.

Steatosis itself does not seem to adversely affect the long-term outcomes of NAFLD,[16] whereas inflammation and hepatocyte injury are the trigger factors for the disease progression to NASH.[17,18] Eupatilin was known as an anti-oxidant, anti-inflammatory, and cytoprotective agent in previous studies.[5-9] And our study showed that eupatilin suppresses hepatic inflammation, oxidative stress and ER stress. Although effect of eupatilin was not enough to eradicate development of NASH, eupatilin is still an attractive agent for NASH because of following reasons. First, many agents which

reduce the liver injury attributed the prevention of NASH in previous studies.[19-22] And many measured values of this study showed dose-dependent pattern. Therefore, more than 100mg/kg dose of eupatilin might have protective effect against development of NASH. Second, *Shin PH et al* reported that cyanidin and PPAR agonists might have synergistic effects against NASH-related oxidative stress.[23] Eupatilin could be used as one part of combination therapy against NASH. For these reasons, further studies about eupatilin as a treatment option against NASH should be considered.

In summary, eupatilin was ineffective to use as a preventative agent for steatohepatitis in mice. Despite of this limitation, it was demonstrated that eupatilin attenuated the hepatocyte damage by suppressing of inflammation, oxidative stress, and/or ER stress. Because hepatic injury was thought to be essential process on development of NASH, further studies are needed to reassess the value of eupatilin in NASH.

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요약 (국문 초록)

배경: 비알코올성 지방간염의 병인은 명확히 밝혀진바 없다. 현재 가장 널리 받아들여지는 가설은 과도한 지방 침착에 이은 간세포 손상으로 간세포 손상은 산화 스트레스, 비정상적인 사이토카인, 미토콘드리아 이상, 소포체 스트레스 등에 의해 발생한다. Eupatilin은 *Artemisia asiatica*에서 추출된 물질로 항산화, 항염증 및 세포 보호 역할을 하는 것으로 알려져 있다. 이에 본 연구에서는 쥐 모델에서 eupatilin이 비알코올성 지방간염의 발생을 예방할 수 있는지 확인하고자 한다.

방법: C57BL/6에 8주 동안 methionine choline 결핍 식이를 제공하고 eupatilin 투약 (50 또는 100 mg/kg 체중 당) 여부에 따라 비알코올성 지방간염의 발생이 억제되는지 확인하고 그 기전을 분석한다.

결과: Eupatilin은 비알코올성 지방간염의 쥐 모델에서 간내 염증, 산화 스트레스, 소포체 스트레스를 억제하여 간 손상을 줄이고 조직학적인 개선을 가져온다. 특히 비알코올성 지방간 질환 활성도 지표 (NAS)를 통계적으로 유의하게 감소시키나 비알코올성 지방간염의 진단 기준인 5 이하로의 호전은 관찰되지 않았다.

결론: Eupatilin에 의한 산화 스트레스와 소포체 스트레스 감소는 비알코올성 지방간염의 발생을 완전히 차단하기에 충분하지 않았다.

하지만 eupatilin의 간세포 보호 기능으로 NAS의 호전을 확인할 수 있었다. 추후 비알코올성 지방간염에서 eupatilin의 예방 효과를 개선시킬 수 있는 추가 연구가 필요하다.

주요어: 비알코올성 지방간, 지방간, eupatilin, 지방질 생합성, 소포체 스트레스, 산화 스트레스

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