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Effects of Quercetin Nanoemulsion on Cholesterol Efflux and MicroRNA-33/34a Expression in the Liver of Mice Fed with a High-Cholesterol Diet

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ABSTRACT: Quercetin is a flavonoid widely present in plants; despite its beneficial physiological activity, it exhibits considerably low bioavailability. Nanoemulsion technology is used for improving the bioavailability of lipophilic phenolic compounds. This study aimed to investigate the potential effects of quercetin nanoemulsion (QN) on regulating the microRNA (miR)-33/34a pathway involved in cholesterol efflux in the liver of mice fed with a high-cholesterol (HC) diet. Subsequently, C57BL/6J mice were divided into four groups and fed a normal chow diet, HC diet supplemented with 1% cholesterol and 0.5% cholic acid, or HC diet supplemented with 0.05% QN or 0.1% QN for 6 weeks. Serum and hepatic lipid profiles were assayed using commercial enzymatic kits. Gene expression and miR levels were quantified using real-time quantitative reverse transcription polymerase chain reaction, and adenosine monophosphate-activated protein kinase (AMPK) activity was measured using an AMPK Kinase Assay kit. QN supplementation improved serum and liver lipid profiles. QN upregulated the mRNA levels of adenosine triphosphate (ATP)-binding cassette subfamily A1, ATP-binding cassette subfamily G1, and scavenger receptor class B type 1, which are related to cholesterol efflux. In the QN group, the hepatic AMPK activity increased, whereas miR-33, and miR-34a expression levels decreased. These results suggest that QN may enhance cholesterol efflux, at least partly through modulating AMPK activity and miR-33/34a expression in the liver.

Keywords: AMP-activated protein kinase, cholesterol, microRNAs, quercetin

INTRODUCTION

Dyslipidemia refers to an imbalance of blood lipids, including hypercholesterolemia, hypertriglyceridemia, high low-density lipoprotein cholesterol (LDL-C) levels, and low high-density lipoprotein cholesterol (HDL-C) levels, due to abnormal lipoprotein metabolism (Wald and Law, 1995). Particularly, an excessive increase in blood cholesterol levels is a risk factor contributing to the development of cardiovascular disease (CVD) accompanied by atherosclerosis (Stone et al., 2014). Therefore, improving dyslipidemia for CVD prevention and treatment is significant.

The liver is a key metabolic organ for regulating cholesterol homeostasis and lipid metabolism; it is responsible for various metabolisms in the body. Adenosine monophosphate-activated protein kinase (AMPK), a phosphorylation enzyme that regulates cholesterol and fatty acid metabolism, plays a role in maintaining cellular energy homeostasis (Hardie et al., 2012). In particular, activated AMPK increases cholesterol efflux capacity (Li et al., 2010; Kemmerer et al., 2016). Lipid transporters, including adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), and scavenger receptor class B type 1 (SR-B1), mediate cholesterol efflux to HDL and facilitate cholesterol flow into the liver (Rosenson et al., 2012). AMPK activates ABCA1 expression in human macrophages and regulates ABCG1-mediated oxysterol efflux in endothelial cells (Li et al., 2010; Kemmerer et al., 2016). A recent study reported that AMPK activation enhances the antiatherosclerotic effect by increasing hepatic SR-B1 and macrophage ABCA1/ABCG1 expression in apoE-deficient mice (Ma et al., 2017).

Quercetin, a flavonoid widely present in plants, is a bioactive compound that possesses strong antioxidant properties (Boots et al., 2008). Moreover, it reportedly has several physiologically active functions, including antioxi-

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dant, anticancer, antiaging, antiviral, anti-obesity, and anti-inflammatory effects (Jung et al., 2013; Wang et al., 2022). Despite quercetin's beneficial physiological activity, its application may be limited by its low solubility and absorption rate (Gao et al., 2009; Kakran et al., 2011). Particularly, owing to its high solubility, permeability, and absorption, oil-in-water (O/W) nanoemulsion, a known food processing technology, is used for improving the bioavailability of lipophilic phenolic compounds (Lohith Kumar and Sarkar, 2018; Liu et al., 2019). We previously formulated quercetin nanoemulsion (QN) by O/W nanoemulsification and observed it to be more effective than quercetin as a raw material in hypocholesterolemic activity through hepatic bile acid synthesis and fecal cholesterol excretion regulation (Son et al., 2019). Here, we aimed to investigate the effects of QN on the mechanisms underlying its regulatory action, focusing on microRNA (miR)-33 and miR-34a expression related to cholesterol efflux in the liver of mice fed with a high-cholesterol (HC) diet.

MATERIALS AND METHODS

QN preparation

QN was supplied by the Korea Food Research Institute and used as O/W nanoemulsion containing quercetin formed via complexation and self-assembly with Tween 80 (Sigma-Aldrich Corp.), caprylic/capric triglyceride (Captex® 355, Abitec Corp.), sodium alginate, and soybean lecithin (IFC Solutions, Inc.), as described in our previous study (Son et al., 2019).

Animals and diet

Six-week-old male C57BL6/J mice were purchased from DooYeol Biotech and housed individually under a constant temperature (22±2°C) and humidity (55±5%) and a controlled 12-h light/dark cycle. After acclimatization for 1 week, mice were divided into the following four groups (n=6 mice/group) and fed an experimental diet for 6 weeks: (1) a normal chow (NC) diet (2018S Rodent Diet, Harlan Teklad, Envigo), (2) HC diet supplemented with 1% cholesterol and 0.5% cholic acid, (3) HC supplemented with 0.05% QN (QNL), or (4) HC supplemented with 0.1% QN (QNH). The experimental diet was formulated on the basis of the American Institute of Nutrition-76 diet composition (Bieri, 1979); it is presented in Table 1. The NC diet comprised 6.2% fat, 44.2% carbohydrates, and 18.6% protein, and the energy density was 3.1 kcal/g. Mice fasted overnight following the experiment were anesthetized with tiletamine-zolazepam (Zoletil 50, Virbac Laboratories) and xylazine (Rompun, Bayer Korea). Blood samples were obtained via direct puncture from the heart through an abdominal incision. The liver tissue was excised and stored at −70°C until analysis. Animal experiments were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University (IACUC no. 16-047).

Serum metabolite assay

Serum was separated from a whole-blood sample by centrifugation at 1,500 *g* for 20 min at 4°C. The serum levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT), which are liver function indicators, were measured using a commercial enzymatic assay kit (Asan Pharmaceutical Co., Ltd.) following the manufacturer's protocol. Serum triglyceride (TG), total cholesterol (TC), and HDL-C concentrations were determined using a commercial assay kit (Embiel Corp.) using the enzyme colorimetric method. The LDL-C concentration was calculated using Friedewald's formula (Friedewald et al., 1972).

Liver lipid analysis

Total lipids were extracted from the liver tissue (0.5 g) using a mixed solvent of chloroform-methanol (2:1, v/v) according to the modified method of Bligh and Dyer (1959). Hepatic TG and TC concentrations were measured using an enzymatic commercial assay kit as described above.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNA from the liver tissue was extracted using TRIzol reagent (GeneAll Bio-technology), and the RNA concentration was quantified using NanoDrop™ (Thermo Fisher Scientific). mRNA and miRNA expression levels

Table 1. Composition of experimental diets (g/kg)

Components	HC	QNL	QNH
Corn starch	150.0	150.0	150.0
Casein	200.0	200.0	200.0
Sucrose	485.0	484.5	484.0
Corn oil	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0
Mineral mix (AIN-76)	35.0	35.0	35.0
Vitamin mix (AIN-76)	10.0	10.0	10.0
DL-Methionine	3.0	3.0	3.0
Choline bitartrate	2.0	2.0	2.0
Cholesterol	10.0	10.0	10.0
Cholic acid	5.0	5.0	5.0
QN	–	0.5	1.0
Total	1,000	1,000	1,000
Energy density (kcal)	3,579.7	3,576.1	3,572.5
Carbohydrate (% as kcal)	67.1	67.1	67.0
Protein (% as kcal)	20.3	20.4	20.4
Fat (% as kcal)	12.6	12.6	12.6

Diets are based on the AIN-76 diet composition (Bieri, 1979). HC, high-cholesterol; QN, quercetin nanoemulsion; QNL, HC+0.05% QN; QNH, HC+0.1% QN.

Table 2. Primers used for real-time quantitative reverse transcription polymerase chain reaction

Gene	GeneBank no.	Primer sequence (5′-3′)
ABCA1	NM_013453	Forward ACG CTG TAC CTG CCC TAT GT Reverse GCT CCT CGA AAA GGG CGA AA
ABCG1	NM_009593	Forward CAA GTG GTG TCT CTG ATG AA Reverse GCA TTG TCC TTG ACT TAG GA
GAPDH	NM_001289726	Forward CCT CAC CCC ATT TGA TGT TA Reverse GTT CCA GTA TGA CTC CAC TC
SR-B1	BC004656	Forward TC TTC ACT GTC TTC ACG GGC Reverse CA TGA AGG GTG CCC ACA TCT

ABCA1, adenosine triphosphate (ATP)-binding cassette subfamily A1; *ABCG1*, ATP-binding cassette subfamily G1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *SR-B1*, scavenger receptor class B type 1.

were quantified using qRT-PCR as previously described (Jang et al., 2022). Complementary DNAs (cDNAs) for mRNA and miR were synthesized from extracted RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Bioneer) and miRNA cDNA Synthesis Kit with Poly(A) Polymerase Tailing (Applied Biological Materials, Inc.), respectively. The qRT-PCR assays were performed in a thermocycler Rotor-Gene Q (Qiagen) using the GreenStar qPCR Master Mix (Bioneer). Primer sequences used for qRT-PCR are presented in Table 2, and they were designed using the Primer3 online program (Rozen and Skaletsky, 2000). mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase as an endogenous control. Specific primers of miR-33, miR-34a, and U6 were purchased from Applied Biological Materials, Inc. Mature miRNA expression was normalized to U6 expression as a reference control. The formal delta-delta Ct method was used for calculating normalized mRNA and miR expression levels in each sample (Livak and Schmittgen, 2001).

AMPK activity assay

AMPK activity was analyzed using the AMPK Kinase Assay kit (CycLex) according to the manufacturer's instructions, as previously described (Lee et al., 2011). Briefly, samples were incubated for 30 min at 30°C on substrate peptide-coated plates corresponding to mouse insulin receptor substrate-1 (IRS-1). AMPK activity was measured via IRS-1 Ser 789 phosphorylation using anti-mouse phospho-Ser 789 IRS-1 monoclonal antibody and peroxidase-coupled anti-mouse IgG. Absorbance was measured at 450 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific) and normalized to protein content. The protein content was measured using a Pierce™ Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific). AMPK activity values were indicated as fold change relative to the HC group.

Statistical analysis

Results were statistically analyzed using IBM SPSS Statistics 25 (IBM Corp.). Values were obtained from six animals per group and expressed as means±standard error. Significant differences in the four groups (NC, HC, QNL, and QNH) were verified using one-way analysis of variance and post-hoc Tukey's multiple range test. $P<0.05$ was considered statistically significant.

RESULTS

Body weight, food intake, energy intake, and serum AST and ALT activities

Body weight, food intake, energy intake, liver weight, and serum AST/ALT values after a 6-week supplementation with the experimental diet are shown in Table 3. Final body weight and weight gain were not significantly different among the NC, HC, QNL, and QNH groups. The HC group had significantly increased food and energy intakes than the NC group ($P<0.05$); however, no significant differences were observed among the experimental groups. Owing to HC-induced hepatomegaly, the HC group showed a significantly increased liver weight than the NC group ($P<0.05$); however, it significantly decreased in the QN groups. To investigate the possible contribution of the QN diet to liver damage, serum AST, and ALT activities were measured. HC-diet-induced serum AST and ALT activities were significantly higher than those of the NC group but significantly lower in the QNH group ($P<0.05$), suggesting that they alleviated liver damage.

Effects of QN on serum and hepatic lipid profiles

Serum and liver lipid profiles following the 6-week QN supplementation are presented in Table 4. Serum TG, TC, and LDL-C concentrations in the HC group were significantly higher by 1.77-, 1.72-, and 5.51-fold than those in the NC group, respectively ($P<0.05$). Serum TG, TC, and LDL-C concentrations were significantly lower in the QNL (27.9, 10.7, and 18.7%) and QNH (34.8, 18.2, and 33.3%) groups than those in the HC group, respectively ($P<0.05$). In contrast, the HDL-C concentration was significantly higher by 1.51- and 1.60-fold in the QNL and QNH groups, respectively ($P<0.05$), showing statistical significance. The HC group had significantly higher hepatic lipid, TG, and TC concentrations than the NC group ($P<$

Table 3. Physiological variables and serum AST and ALT levels in mice fed with NC, HC, QNL, and QNH diets for 6 weeks

Variables	NC	HC	QNL	QNH
Initial body weight (g)	21.3±0.33	21.3±0.29	21.3±0.22	21.3±0.21
Final body weight (g)	25.2±0.10	25.0±0.13	24.7±0.14	24.7±0.21
Body weight gain (g/6 wk)	3.87±0.26	3.52±0.30	3.42±0.27	3.43±0.25
Food intake (g/d)	3.80±0.07^{b}	4.74±0.08^{a}	4.73±0.06^{a}	4.63±0.12^{a}
Energy intake (kcal/d)	11.7±0.24^{b}	16.9±0.28^{a}	16.9±0.23^{a}	16.6±0.44^{a}
Liver weight (g/100 g body weight)	4.10±0.16^{c}	9.49±0.11^{a}	8.53±0.19^{b}	8.49±0.35^{b}
Serum AST (IU/L)	57.9±1.40^{c}	81.9±2.20^{a}	79.3±2.08^{ab}	72.5±2.71^{b}
Serum ALT (IU/L)	9.62±0.59^{c}	39.5±0.76^{a}	38.6±0.92^{ab}	35.1±1.61^{b}

Values are expressed as mean±SEM (n=6). Means in a row with superscripts (a-c) without a common letter differ, $P<0.05$. AST, aspartate aminotransferase; ALT, alanine aminotransaminase; NC, normal chow; HC, high-cholesterol; QN, quercetin nanoemulsion; QNL, HC+0.05% QN; QNH, HC+0.1% QN.

Table 4. Serum and hepatic lipid profiles in mice fed with NC, HC, QNL, and QNH diets for 6 weeks

Metabolites	NC	HC	QNL	QNH
Serum (mmol/L)				
TG	0.68±0.03^{b}	1.20±0.08^{a}	0.86±0.06^{b}	0.78±0.08^{b}
TC	2.73±0.11^{c}	4.69±0.14^{a}	4.19±0.10^{b}	3.83±0.13^{b}
HDL-C	2.01±0.03^{a}	1.33±0.03^{c}	1.51±0.05^{b}	1.60±0.15^{b}
LDL-C	0.51±0.05^{d}	2.82±0.10^{a}	2.29±0.05^{b}	1.88±0.12^{c}
Liver				
Total lipid (mg/g)	26.1±1.22^{d}	93.0±6.61^{a}	72.5±3.32^{b}	54.2±3.87^{c}
TG (μmol/g)	3.85±0.39^{b}	11.8±0.80^{a}	5.08±0.47^{b}	4.09±0.34^{b}
TC (μmol/g)	6.72±0.44^{d}	22.3±0.35^{a}	18.4±0.39^{b}	16.2±0.22^{c}

Values are expressed as mean±SE (n=6). Means in a row with superscripts (a-d) without a common letter differ, $P<0.05$. NC, normal chow; HC, high-cholesterol; QN, quercetin nanoemulsion; QNL, HC+0.05% QN; QNH, HC+0.1% QN; TG, serum triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

0.05). In contrast, hepatic lipid, TG, and TC concentrations were significantly lower in the QNL (22.1, 56.8, and 17.6%) and QNH (41.7, 65.2, and 27.5%) groups than those in the HC group, respectively ($P<0.05$).

Effects of QN on the gene expression related to cholesterol efflux in the liver

We investigated the effects of QN on the expression of genes involved in hepatic cholesterol efflux. The HC diet significantly downregulated the gene expression of ABCA1, ABCG1, and SR-B1, which are transporters involved in hepatic cholesterol efflux, compared with the NC diet ($P<0.05$). In contrast, the gene expression of ABCA1, ABCG1, and SR-B1 was upregulated in the QNL (1.48-, 1.32-, and 1.62-fold) and QNH (1.74-, 1.56-, and 1.87-fold) groups compared with that in the HC group, respectively ($P<0.05$; Fig. 1).

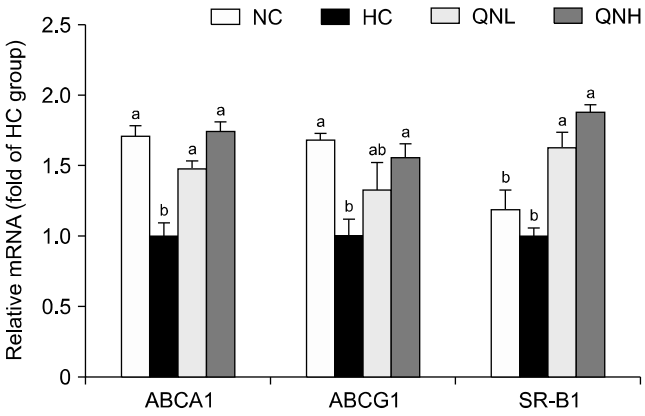

Fig. 1. Expression of genes related to cholesterol efflux in the liver of mice fed with normal chow (NC), high-cholesterol (HC), HC+0.05% QN (QNL), and HC+0.1% QN (QNH) diets for 6 weeks. The mRNA levels of adenosine triphosphate (ATP)-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1), and scavenger receptor class B type 1 (SR-B1) are measured using real-time quantitative reverse transcription polymerase chain reaction. Values are expressed as mean±SE (n=6). Different letters (a,b) indicate significant differences among the four groups (NC, HC, QNL, and QNH groups) at $P<0.05$. QN, quercetin nanoemulsion.

Effects of QN on hepatic AMPK activity

The effects of QN on AMPK activity, which plays a significant role in cholesterol homeostasis in the liver, were investigated. The hepatic AMPK activity significantly decreased by 35.9% in the HC group compared with that in the NC group ($P<0.05$); however, it significantly increased by 1.65-fold in the QNH group compared with

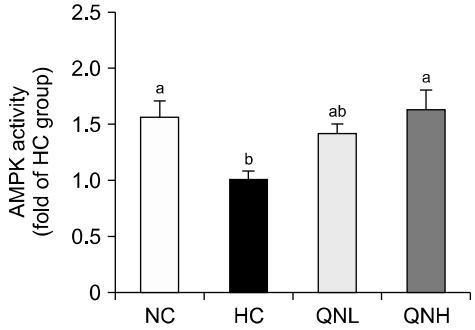

Fig. 2. Adenosine monophosphate-activated protein kinase (AMPK) activity in the liver of mice fed with normal chow (NC), high-cholesterol (HC), HC+0.05% QN (QNL), and HC+0.1% QN (QNH) diets for 6 weeks. Values are expressed as mean±SE (n=6). Different letters (a,b) indicate significant differences among the four groups (NC, HC, QNL, and QNH groups) at $P<0.05$. QN, quercetin nanoemulsion.

that in the HC group ($P<0.05$; Fig. 2).

Effects of QN on hepatic miR-33 and miR-34a expression

The expression of miR-33 and miR-34a, which regulate liver lipid metabolism, was investigated to confirm the mechanism of miR regulation by QN. The hepatic miR-33 expression level was significantly increased by 1.66-fold in the HC group compared with that in the NC group ($P<0.05$); however, it was significantly suppressed by 40.4% and 44.4% in the QNL and QNH groups, respectively, compared with that in the HC group ($P<0.05$; Fig. 3A). The hepatic miR-34a expression level was significantly increased by 10.3-fold in the HC group compared with that in the NC group; however, it was significantly suppressed by 31.3% and 39.4% in the QNL and QNH groups, respectively, compared with that in the HC group ($P<0.05$; Fig. 3B).

DISCUSSION

In this study, the HC diet induced dyslipidemia by increasing blood cholesterol, TG, and LDL-C levels as well as significantly increased hepatic lipid accumulation. This study aimed to elucidate the effects of QN on serum and liver lipid profiles in HC diet-fed mice and understand the regulatory mechanisms underlying miR involvement in hepatic lipid metabolism. In this study, the serum TG, TC, and LDL-C concentrations in QN diet-fed mice significantly decreased compared with those in HC diet-fed mice; however, HDL-C concentrations were significantly higher. Moreover, hepatic lipid profiles were significantly decreased by QN supplementation. Several studies have reported that quercetin inhibits serum and hepatic lipid level increases, thereby implying its antilipidemic properties (Jeong et al., 2012; Jung et al., 2013; Li et al., 2013). Consistent with our previous results (Son et al., 2019), our current findings indicate that QN, a quercetin-mediated nanoemulsion, reduces serum, and liver lipid levels, suggesting that it improves dyslipidemia.

We previously reported that QN more effectively upregulated the expression levels of cholesterol 7 alpha-hydroxylase, liver X receptor alpha, and ATP-binding cassette transporters G5/G8, which are involved in cholesterol excretion in the liver of hypercholesterolemic rats, than quercetin in HC diet-fed mice (Son et al., 2019). In this study, we investigated hepatic regulatory mechanisms by which QN reduces liver and serum lipid levels and increases HDL-C levels in HC diet-fed mice. QN increased the expression of ABCA1, ABCG1, and SR-B1, which mediate cholesterol efflux into HDL, as well as increased AMPK activity. ABCA1, ABCG1, and SR-B1 expression are increased by AMPK activation, suggesting that AMPK activation helps enhance HDL maturation (Ma et al., 2017). Quercetin reportedly enhances ABCA1 and ABCG1 expression and cholesterol efflux in macrophages (Chang et al., 2012; Cui et al., 2017). Our results suggest that QN is potentially beneficial in terms of increasing HDL-C concentrations as it positively feedbacks AMPK activation and cholesterol efflux in the liver.

miRNAs are small non-coding RNA molecules comprising approximately 22 nucleotides; they are involved in gene expression in tissues with high metabolic activity, including liver and adipose tissues (Green et al., 2016). miR-33 and miR-34a are key regulators of cholesterol efflux and atherosclerosis (Goedeke et al., 2013; Xu et al., 2020). Particularly, miR-33 suppresses ABCA1 expression and reduces HDL-C levels (Rayner et al., 2011). miR-34a directly binds to the 3' untranslated region of ABCA1 and

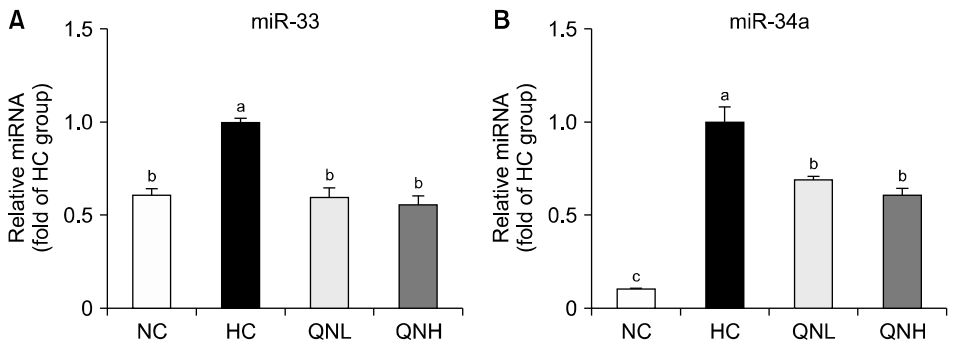

Fig. 3. microRNA (miR)-33 (A) and miR-34a (B) expression in the liver of mice fed with normal chow (NC), high-cholesterol (HC), HC+0.05% QN (QNL), and HC+0.1% QN (QNH) diets for 6 weeks. Values are expressed as mean±SE (n=6). Different letters (a-c) indicate significant differences among the four groups (NC, HC, QNL, and QNH groups) at $P<0.05$. QN, quercetin nanoemulsion.

ABCG1, and miR-34a inhibition promotes macrophage cholesterol efflux by inducing ABCA1 and ABCG1 expression (Xu et al., 2020). Additionally, treatment with an miR-34a inhibitor stimulates the AMPK phosphorylation pathway in L02 hepatocytes and mouse liver tissues (Ding et al., 2015). Therefore, the roles of miR-33, and miR-34a in cholesterol metabolism may be potential biomarkers in atherosclerosis. Recently, Kim et al. (2022) have reported that quercetin and its metabolite, isorhamnetin, suppress liver DNA damage via miR-34a downregulation in HepG2 cells. However, QN's effects on the regulatory mechanisms of miR-33 and miR-34a in the liver of HC diet-fed mice are yet to be elucidated. In this study, we first observed that QN suppresses miR-33 and miR-34a expression in the liver of HC diet-fed mice. Therefore, it can be speculated that QN regulates hepatic cholesterol efflux, which is partly related to the hepatic miR-33/34a pathways.

In conclusion, our findings indicate that QN improves liver and serum lipid levels in HC diet-fed mice and enhances HDL-C through AMPK activation and the regulation of gene expression involved in cholesterol efflux. Furthermore, this study is the first to report that the beneficial effects of QN on hepatic cholesterol efflux are partly associated with miR-33 and miR-34a pathway regulation (Fig. 4). Therefore, it suggests that QN is beneficial as a potential substitute strategy for preventing CVD development, including dyslipidemia, and atherosclerosis. QN is believed to help improve the bioavailability of quercetin, which has low solubility and absorption rate, and studies on changes in its absorption rate *in vivo* should be conducted in the future. Additionally, since this study is a single study of QN and the scope of application is very limited, future research on clinical application and safety through comparative studies of quercetin and QN is necessary.

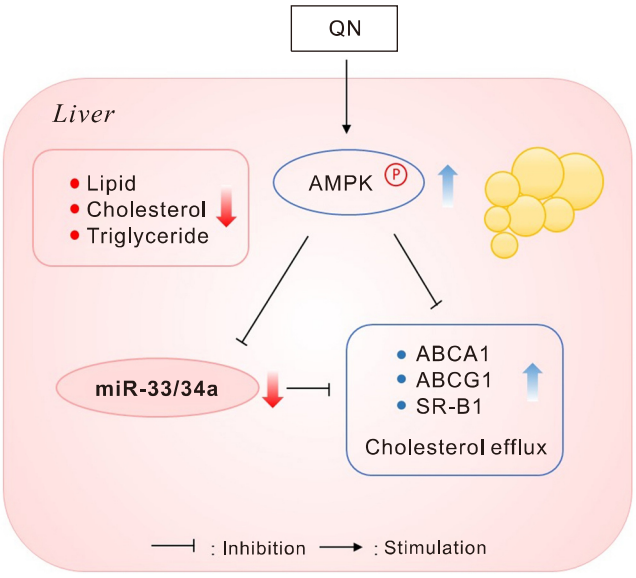

Fig. 4. Schematic diagram showing possible mechanisms by which quercetin nanoemulsion (QN) regulates hepatic lipid metabolism and the microRNA (miR)-33/34a pathways. AMPK, adenosine monophosphate-activated protein kinase; ABCA1, adenosine triphosphate (ATP)-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; SR-B1, scavenger receptor class B type 1.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: YK. Analysis and interpretation: MSL. Data collection: MSL. Writing the article: MSL. Critical revision of the article: YK. Final approval of the article: all authors. Statistical analysis: MSL. Obtained funding: YK. Overall responsibility: YK.

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