Research Article

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Mirtoselect, an anthocyanin-rich bilberry extract, attenuates non-alcoholic steatohepatitis and associated fibrosis in ApoE*3Leiden mice

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Background & Aims: Anthocyanins may have beneficial effects on lipid metabolism and inflammation and are demonstrated to have hepatoprotective properties in models of restraint-stress- and chemically-induced liver damage. However, their potential to protect against non-alcoholic steatohepatitis (NASH) under conditions relevant for human pathogenesis remains unclear. Therefore, we studied the effects of the standardised anthocyanin-rich extract Mirtoselect on diet-induced NASH in a translational model of disease.

Methods: ApoE*3Leiden mice were fed a Western-type cholesterol-containing diet without (HC) or with 0.1% (w/w) Mirtoselect (HCM) for 20 weeks to study the effects on diet-induced NASH.

Results: Mirtoselect attenuated HC-induced hepatic steatosis, as observed by decreased macro- and microvesicular hepatocellular lipid accumulation and reduced hepatic cholesteryl ester content. This anti-steatotic effect was accompanied by local anti-inflammatory effects in liver, as demonstrated by reduced inflammatory cell clusters and reduced neutrophil infiltration in HCM. On a molecular level, HC diet significantly induced hepatic expression of pro-inflammatory genes *Tnf*, *Emr1*, *Ccl2*, *Mpo*, *Cxcl1*, and *Cxcl2* while this induction was less pronounced or significantly decreased in HCM. A similar quenching effect was observed for HC-induced pro-fibrotic genes, *Acta2* and *Col1a1* and this anti-fibrotic effect of Mirtoselect was confirmed histologically. Many of the pro-inflammatory and pro-fibrotic parameters positively correlated with intrahepatic free cholesterol levels. Mirtoselect significantly reduced accumulation and crystallisation of intrahepatic free cholesterol, providing a possible mechanism for the observed hepatoprotective effects.

Conclusions: Mirtoselect attenuates development of NASH, reducing hepatic lipid accumulation, inflammation and fibrosis, possibly mediated by local anti-inflammatory effects associated with reduced accumulation and crystallisation of intrahepatic free cholesterol.

Keywords: Anthocyanins; Bilberry; Cholesterol crystals; Fibrosis; Free cholesterol; Non-alcoholic steatohepatitis; Polyphenols; Steatosis.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; E3L, ApoE*3Leiden; HC, high-cholesterol control group; HCM, high-cholesterol + Mirtoselect group; REF, reference group; CK18, cytokeratin 18.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in Western countries [1,2]. It constitutes a spectrum of liver injury, ranging from the clinically benign intrahepatic accumulation of lipids (steatosis), to the more progressive non-alcoholic steatohepatitis (NASH). In addition to hepatic lipid accumulation, NASH is characterised by hepatic inflammation, i.e. infiltration of immune cells [3] and can further progress to fibrosis, cirrhosis and hepatocellular carcinoma. Although the mechanisms by which NASH progresses are not completely understood, it is thought that dysregulation of cholesterol homeostasis and subsequent accumulation of free (unesterified) cholesterol are linked to the pathogenesis of NASH in humans (reviewed in reference [4]). In line with this notion, emerging experimental evidence implicates free cholesterol as a potential trigger of inflammation [5] as well as a possible driving factor in the development of fibrosis [6,7]. A recent study in experimental and human NASH revealed that intrahepatic accumulation of free cholesterol can lead to the formation of cholesterol crystals in hepatocyte lipid droplets, which may form an important trigger for the progression of simple steatosis to NASH [8].

The anthocyanins, a subclass of the polyphenols, comprise a large group of bioactive compounds that are considered to have many health-promoting effects [9], including cholesterol-lowering [10,11] and anti-inflammatory effects [12] which may mediate potential hepatoprotective properties [13]. Here we studied the effects of the standardised anthocyanin-rich bilberry (*Vaccinium myrtillus* L.) extract Mirtoselect on the development of

NASH. This extract has been demonstrated to reduce circulating makers of inflammation in humans [14,15] and has beneficial effects in restraint-stress- [16,17] and chemically-induced [18] models of liver damage. However, its hepatoprotective potential in diet-induced metabolic inflammation and liver disease is unclear. Therefore we studied effects of Mirtoselect on NASH in ApoE*3Leiden (E3L) mice, a translational model of disease [19]. These mice develop diet-induced dyslipidaemia and inflammation on a high-fat/high-cholesterol diet [20], and ultimately develop NASH with fibrosis. Earlier studies have shown that E3L mice are sensitive to nutritional [21] and pharmacological [19] interventions, and show human-like responses to hypolipidaemic compounds [19,22].

Combined histological, biochemical, and gene expression analyses revealed that Mirtoselect reduces development of NASH, attenuating both steatosis and inflammation as well as the development of hepatic fibrosis. These effects were associated with a reduction in hepatic free cholesterol accumulation and cholesterol crystal formation.

Materials and methods

Animal experiments

Experiments were approved by an independent Animal Care and Use Committee and were in compliance with European Community specifications regarding the use of laboratory animals. E3L mice were used because they allow for the study of diets and nutrients on lipids (including cholesterol) and liver inflammation [19,23,24].

Female E3L mice were fed a Western-type diet (15% cocoa butter, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch and 6.2% cellulose; diet-T; AB-Diets, Woerden, The Netherlands), supplemented with 1% (w/w) cholesterol (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 20 weeks. The study included a 4-week run-in during which all mice received this diet, after which they were matched for plasma cholesterol and triglycerides into three experimental groups (n = 15/group). Control animals (HC) continued to receive the Western-type diet for the remainder of the study, while Mirtoselect-treated animals (HCM) received the HC diet with addition of 0.1% (w/w) Mirtoselect (Indena S.A.S., Paris, France). This standardised bilberry (*Vaccinium myrtillus* L.) extract contains 36% anthocyanins. An ageing reference group (REF) received the same Western-type diet mentioned above, but without cholesterol supplementation. Food intake and body weight were monitored throughout the study. Every 4 weeks, blood samples were collected via tail vein bleeding after a 4 h fast for isolation of EDTA plasma. Animals were sacrificed by CO_2 asphyxiation after 16 weeks of dietary treatment to collect livers. The medial lobe was fixed in formalin and embedded in paraffin for histological analysis of NASH and the left lobe was snap frozen in liquid nitrogen and stored at −80 °C for cryosectioning, liver lipid- and mRNA-expression analyses.

Histological, biochemical and hepatic gene expression analyses

A detailed description of (immuno)histological, biochemical, and gene expression analyses is provided in (Supplementary Material and methods). Briefly, development of NASH was assessed histologically using an adapted grading method for human NASH [25,26]. Plasma lipids were determined with commercially available enzymatic assays and liver lipids were analysed by HPTLC, as described previously [27]. Hepatic gene expression analyses were performed by RT-PCR, using TaqMan® Gene Expression Assays (Life Technologies, Bleiswijk, The Netherlands) and changes in gene expression were calculated using the comparative Ct (ΔΔCt) method, expressed as fold-change relative to REF. Illumina microarray analysis of hepatic gene expression was performed following established normalisation and quality control protocols followed by gene enrichment analysis across pathways and biological processes as described [23]. p65-NFκB activity was determined in liver homogenates by DNA-binding ELISA (TransAM® p65-NFκB Chemi Kit, Active Motif, La Hulpe, Belgium) according to manufacturer's instructions and as described [26].

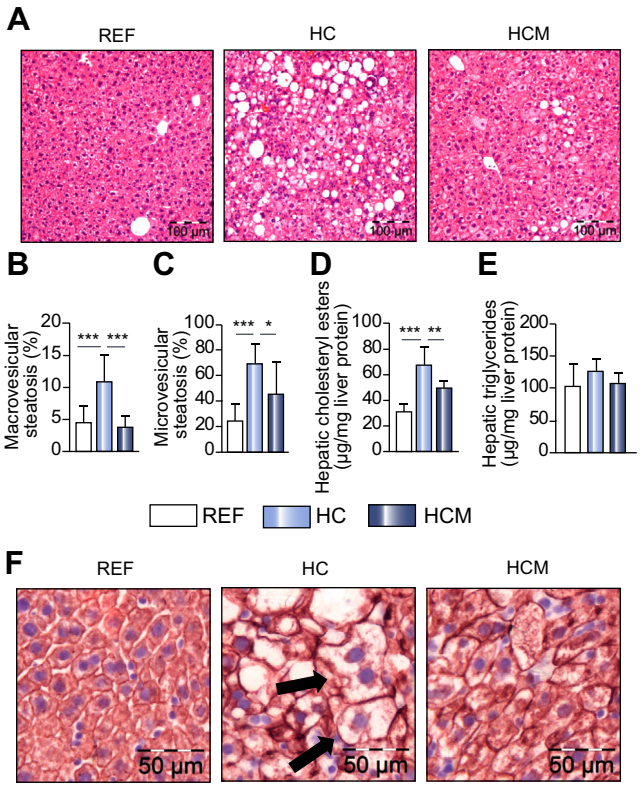

Fig. 1. Mirtoselect attenuates hepatic steatosis in cholesterol-fed E3L mice. (A) Representative photomicrographs of liver sections of reference (REF), high-cholesterol control (HC) and Mirtoselect-treated mice (HCM). Mirtoselect attenuated HC diet-induced macrovesicular (B) and microvesicular (C) steatosis. (D) Hepatic steatosis in HC mice was mainly attributable to accumulation of cholesteryl esters, the build-up of which was decreased by Mirtoselect. (E) Hepatic triglycerides tended to accumulate in HC, which was not observed in HCM. (F) In HC-fed animals, some hypertrophic cells were CK18-deficient compared with neighbouring cells (arrows); presence of these cells was reduced in HCM. REF: non-cholesterol-fed reference, HC: high-cholesterol control, HCM: high-cholesterol + Mirtoselect. Data are mean ± SD. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 compared with HC.

Statistical analyses

All data are presented as mean ± SD. Statistical analyses were performed using SPSS software (version 22, IBM, Armonk, USA). For normally distributed variables, significance of differences between groups was tested by one-way ANOVA, with Dunnett's multiple comparison post-hoc test to compare HC *vs.* REF and HC *vs.* HCM. In case of heterogeneity between groups, variables were analysed by ANOVA using Brown-Forsythe for differences between groups with Dunnett's T3 post-hoc test. Non-normally distributed variables were tested by non-parametric Kruskal-Wallis test followed by Mann-Whitney *U*. A p value <0.05 was considered statistically significant.

Results

Mirtoselect attenuates hepatic steatosis and hepatocellular damage

Treatments were well tolerated and there was no effect of Mirtoselect on food intake or body weight (Supplementary Fig. 2A–B). HC-feeding induced hepatosteatosis relative to REF, as observed histologically by a non-zonal accumulation of lipid macrovesicles and microvesicles in hepatocytes (Fig. 1A).