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学位の種類	博士（医科学）
学位記番号	博甲第　９３３５　号
学位授与年月	令和元年１２月３１日
学位授与の要件	学位規則　第４条第１項該当（昭和２８年４月１日文部省令第９号）
審査組織	グローバル教育院
学位論文題目	Alleviative Effect of Isorhamnetin and Its Derivatives on Nonalcoholic Steatohepatitis （イソラムネチンおよびその類縁化合物による非アルコール性脂肪性肝炎に対する緩和効果）

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Abstract of thesis

Nonalcoholic steatohepatitis (NASH) is the most severe and progressive form of nonalcoholic fatty liver disease which affects people who do not or little drink alcohol. The pathogenesis of NASH is mostly explained by ‘Two-hit hypothesis’ representing intra-hepatic lipid accumulation due to metabolic syndromes as the ‘first hit’ and followed by the ‘second hit’ resulting in increased inflammation and liver injury with fibrosis. A build-up of fat in the liver so-called steatosis is usually considered benign, but it may progress to more severe pathologic condition over the course of several years when it is combined with inflammation, injury, and fibrosis resulting in NASH which can in turn potentially progress to end-stage liver diseases such as cirrhosis and hepatocellular carcinoma. The fact that NASH pathologic features are reversible which are manifested just before irreversible end-stage liver diseases, it emphasizes the importance and urgency of finding a treatment of NASH to prevent patients to progress into advanced disease steps. However, so far, there are still no drugs approved to treat NASH.

Flavonoids are polyphenols widely presented in fruits, vegetables, stems, seeds, etc., and have biological activities against metabolic diseases in addition to their strong antioxidant activities. Quercetin is widely studied

flavonoid with potentials against carcinoma, inflammation, fibrogenesis, and oxidative stress. Isorhamnetin is an immediate metabolite of quercetin, also found in various plant extracts and plant-derived products. It has been shown to (1) prevent oxidative stress in HepG2 cells, (2) inhibit lung and breast cancer cell proliferation, (3) improve inflammatory bowel disease, and (4) repress adipogenesis in 3T3-L1 cells. Given that these are closely related to the pathogenesis of NASH, the author hypothesized that isorhamnetin may possess the alleviative effect on NASH.

Thus, firstly the author investigated the effect of isorhamnetin on NASH pathologic features in the liver. NASH was induced in C57BL/6 mice and treated with isorhamnetin orally. Liver and serum were isolated from experimental groups for biometrical, biochemical, histological, gene expression, and microarray analysis. Great number of genes mainly involved in lipid metabolism, oxidation reduction process, and fatty acid metabolism were altered following the induction of NASH. The number of altered genes were remarkably decreased by the isorhamnetin treatment. Consistently, genes involved in fatty acid metabolism, steroid biosynthesis, and PPAR signaling pathway were invariably decreased. In addition, isorhamnetin treatment reduced intrahepatic lipid accumulation associated with lower triglycerides content and inhibited *de novo*lipogenic pathway in NASH-induced mice. Liver injury markers in serum were consistently improved when treated with isorhamnetin compared with their non-treated NASH-induced littermates. Along with the anti-steatosis effect, isorhamnetin reduced fibrogenic marker gene expressions accompanied with the reduced area of collagen deposition on liver sections. In addition, a number of apoptotic cells were significantly decreased after the treatment. Analysis in adipose tissue revealed the infiltration of macrophages in NASH-induced mice showing a chronic inflammatory state while the treatment with isorhamnetin alleviated this inflammatory condition in adipose tissue.

The author next sought to investigate the structure-activity relationship of methyl group by which isorhamnetin differs from quercetin on the development of fibrosis. Previous study showed that the most part of absorbed quercetin is found in its methylated form, which is chemically equivalent to isorhamnetin and is maintained in plasma longer than quercetin. It strongly implies a potential role of isorhamnetin as a main mediator of beneficial effect of quercetin. Toreveal the importance of methyl group of isorhamnetin, the author used the synthesized five different mono-methylated derivatives of quercetin namely isorhamnetin, azaleatin, 3-methylquercetin, tamarixetin, rhamnetin. Hepatic fibrosis is initiated primarily by the hepatic stellate cells (HSC). Chronic injury to liver caused by metabolic disorders, alcoholism, viral infections, and NASH can lead to transdifferentiation of HSCs from its quiescent resting state into its activated state characterized by more migratory, proliferative, and contractile myofibroblast-like phenotype. This activated HSCs promotes extracellular matrix (ECM) molecules including different types of collagens leading to development of fibrosis, and further hepatic injuries which are irreversible. Thus, the author used HSCs as an *in vitro* fibrosis model. Fibrosis was induced bytransforming growth factor-β (TGFβ) in rat stellate cells (HSC-T6) and then cells were treated with methylated derivatives at various dose and time. Immunofluorescence staining of collagen type I (Col1) and αSMA, cell proliferation assay, and fibrogenic gene expression analysis were conducted. All derivatives showed antiproliferative effects in dose- and time-dependent manner in HSC-T6 cells. The treatment of 3MQ and RHA reduced the protein level and mRNA expression of*Acta2*(gene encoding αSMA) while 3MQ decreased the levels of *Col1a1*(gene encoding Col1) in TGFβ-induced stellate cells. Each compound had different effects against

pathologic features of fibrosis which suggests that -OH position plays an important role in the regulation of anti-fibrotic activity of compound. Although the molecular mechanism underlying their antifibroticeffect remains to be elucidated, these results demonstrated for the first time that methylation could improve the antifibrotic effect of quercetin.

In conclusion, these findings collectively suggest that isorhamnetin elicits beneficial effect on hallmarks of NASH by improving steatosis, injury, and fibrosis in a novel human like NASH-induced mice. This hepatoprotective effect of isorhamnetin was correlated to the inhibition of *de novo*lipogenicand fibrogenic gene expressions, alleviation of liver triglycerides (TG) content, and diminution of hepatic collagen deposition accompanied with the reduced number of apoptotic hepatocytes. Thus, isorhamnetin can be a novel candidate for the consideration of additional compound in NASH drug development. Moreover, the addition of methyl group on functionally important position may enhance the antifibrotic effect of quercetin. Further evidence with human NASH will be required to understand the effect of isorhamnetin on NASH development.

Abstract of assessment result

【Review】

Non-alcoholic fatty liver disease (NAFLD) is characterized by deposition of lipids in the hepatic parenchyma5% of liver weight in the absence of other conditions. Non-alcoholic steatohepatitis (NASH) is an advanced form of NAFLD. The pathogenesis of NASH is closely associated with inflammation, injury, and fibrosis, which can lead to liver cirrhosis and hepatocellular carcinoma. As the pathogenic feature of NASH is thought to be reversible which is manifested just before irreversible end-stage liver diseases, a treatment of NASH to prevent patients to progress into advanced disease steps is important. However, no drugs have been approved to treat NASH. The applicant investigated the alleviative effect of isorhamnetin, a plant-derived flavonoid, against NASH. The applicant found that treatment of isorhamnetinsignificantly reduced intrahepatic lipid accumulation associated with lower triglycerides content and inhibited *de novo*lipogenic pathway in NASH-induced mice. Isorhamnetin also suppressed the collagen depostion in liver section along with reduced expression of fibrogenic genes and inflammatory condition in adipose tissue. The applicant also revealed the structure-activity relationship on anti-fibrogenic effect in hepatic stellate cell line by using synthesized five different mono-methylated derivatives of quersetin.Thus, isorhamnetin is expected to be a novel candidate for the agent to treat and prevent NASH.

【Result】

The final examination committee conducted a meeting as a final examination on 23October 2019. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded Doctor Philosophy in Medical Science.